Studies on Interaction of Plasminogen with Balb/c 3T3 and SV3T3 Cells in Culture

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1978

(Submitted 2 February 1978)

TO MY PARENTS

Acknowledgments

I wish to thank my friends and fellow graduate students--Roger Koeppe, Mike Ross, Steve Spencer, John Chambers, Diane Kent, John Ruark, Mike Klymkowsky, Dave Agard, and Mel Jones--for sharing their ideas and offering encouragement through some of this work. Special thanks is to be given to Monty Krieger who taught me to be critical in every aspect of scientific investigation.

The Chemistry Department staff is to be thanked, for without their efforts none of this research would have been possible.

I wish to thank Dee Barr for typing the thesis and the manuscripts and for offering me advice and encouragement.

Marlyn Teplitz is to be thanked for teaching me the microbial techniques necessary for much of this work, but more so for sharing her insight and wisdom into many aspects of life.

I wish to thank my parents, to whom this dissertation is dedicated, for their encouragement and also for reinforcing my desires to be formally educated.

The person probably most responsible for the completion of this work is Maura Kiernan. Without her constant help, devotion and encouragement, I would have never made it through those last few months. I hope in years to come I can be as great a help to her.

Finally, I would like to thank Bob Stroud for his courage in undertaking a research project of this type and for his ideas and support.

This work was supported in part by a National Institutes of Health Traineeship.

Abstract

The existence of a specific transformed cell surface-plasminogen interaction which has been implicated in many phenotype changes that occur in cells upon transformation was studied. In addition, the fate of the plasmin in cell culture medium activated by a cell-derived plasminogen activator was investigated.

As an initial step, the plasminogen binding to Balb/c 3T3 and SV3T3 cells was studied using ^{125}I-labeled dog plasminogen. The binding appears to be independent of the protease-dependent morphological change which is exhibited by many transformed cells. The ^{125}I-plasminogen which bound to the SV3T3 cells was completely degraded during three days of incubation to macromolecules which were the same size as the large and small chains of active plasmin, and to smaller fragments including 3-iodo-L-tyrosine. The plasminogen which bound to the 3T3 cells was only partially degraded to 3-iodo-L-tyrosine with intermediate conversion to plasmin-size peptides.

The results of a sublethal cell-surface trypsinization assay, developed to determine whether the processing of plasminogen by cells was a cell-surface phenomenon or an internal process, suggest that the cell-associated plasminogen was primarily bound to the surfaces of the 3T3 and SV3T3 cells while the macromolecular degradation products were inside the cells.

Therefore, the 3T3 and SV3T3 cells bound, endocytosed and degraded serum plasminogen. The overall rate of this processing was

approximately twice as fast for the SV3T3 cells as the 3T3 cells. This binding and processing of plasminogen does not appear to be a specific plasma membrane-plasminogen interaction and is known to be independent of the protease-dependent morphological change.

Essentially none of the plasminogen in the 3T3 growth medium was activated to plasmin, while a significant amount of the plasminogen in the SV3T3 growth medium was activated to plasmin, and subsequently complexed with a serum inhibitor of 47,000 molecular weight. This inhibitor is covalent, forming a hydroxylamine-dissociable bond.

To investigate whether any active plasmin remained in SV3T3 growth medium, a model system of urokinase in situ activation of plasminogen designed to mimic the levels of plasminogen activation observed in a 48-hour incubation of plasminogen with SV3T3 cells was developed. Some of the plasmin in this model system incorporated ^{32}P-labeled diisopropylfluorophosphate, implying that active plasmin exists in SV3T3 cell culture medium and thus may be responsible for the protease-dependent morphological change expressed by these cells and other phenotypic changes which occur upon virus transformation.

Preface

This thesis summarizes work which is reported in detail in reprints and preprints appearing in the Appendices.

Table of Contents

The hypothesis that cancer cells produce degradative enzymes which facilitate their invasive properties is not new. As early as 1925, Fischer (1) noted degradation of fibrin clots in the presence of cultured tumor tissues. Later, it was found that in some cases of prostatic cancer the fibrinolytic capacity of the blood increased (2,3) and that fibrin degradation products are often demonstratable in patients with malignant diseases (4,5).

These observations lead to a series of in vitro studies whose goal was to ascertain whether untransformed cells exposed to proteolytic enzymes took on phenotypic characteristics of virus-transformed cells. Burger (6) first showed that untransformed mouse 3T3 cells subjected to proteolytic enzyme treatment increased their agglutinability by wheat germ agglutinin to levels equivalent to those of virus-transformed cells. This observation was extended to fibroblasts of several different origins whose agglutinability was mediated by lectins of several different sources (7-12). Thus, that protease treatment of untransformed cells increases lectin agglutinability to levels similar to that of transformed cells appears to be a general, reproducible phenomenon.

Viral transformation of fibroblasts is almost invariably accompanied by the loss of the iodinatable plasma membrane LETS (for Large, External Transformation-Sensitive) glycoprotein (13). When untransformed cells are exposed to low concentrations (less than that required to remove the cells from the substratum) of the proteases, trypsin or plasmin, the LETS protein is removed from their cell

surfaces (14-16).

Virus-transformed cells have an increased rate of glucose uptake compared with dense or growing cultures of untransformed cells. In general, the rate of glucose transport is lowest in confluent untransformed cultures, greater in growing cultures, and greatest in cultures of virus-transformed cells (17). When confluent cultures of untransformed chick embryo cells are treated with trypsin (18), plasmin (19), thrombin (20), or collagenase (20), the glucose uptake increases but does not reach the level of uptake observed in chick embryo cells transformed by Rous sarcoma virus (19). Thus, the protease-treated untransformed cells approach but do not exactly mimic the glucose uptake of transformed cells.

Cyclic adenosine 3',5'-monophosphate (cAMP) levels are lower in virus-transformed fibroblast cells than their confluent untransformed counterparts (21). The intracellular level of cAMP appears to influence phenotypic characteristics such as cell motility, transport of nutrients across the plasma membrane, growth in agar, and agglutinability by plant lectins (21,22). Therefore, many of the characteristic phenotypic changes observed upon viral transformation of cells may be secondary consequences of low cAMP levels. Untransformed cells treated with trypsin exhibit a transient decrease in cellular cAMP levels (23-26). So once again protease treatment of untransformed cells produces characteristics similar to virus-transformed cells.

Virus-transformed cells grow to high or indefinite saturation densities in culture, while untransformed cells exhibit density

dependent inhibition of growth (27). Burger observed that confluent mouse 3T3 cells could be released from density-dependent inhibition of growth by treatment with trypsin or pronase (28-30). However, other laboratories have been unable to stimulate cell proliferation of 3T3 cells by protease treatment (31-33). On the other hand, several laboratories have been able to reproducibly stimulate growth of confluent chick embryo fibroblasts by treatment with proteolytic enzymes including trypsin (34,35), pronase (33,34), thrombin (20,36), and collagenase (20). Thus at least in chick embryo fibroblasts, protease treatment bestows growth characteristics similar to those of virus-transformed cells.

So, in terms of agglutinability by plant lectins, loss of external transformation sensitive glycoprotein (LETS protein), increase in glucose uptake, decrease in cAMP levels, and possibly release from density-dependent inhibition of growth, protease treatment of untransformed cells produces phenotypic characteristics common to virus-transformed cells.

In vitro studies were also undertaken to answer the reverse question; that is, if proteases are responsible for the expression of certain phenotypic characteristics of virus-transformed cells, what effects do protease inhibitors have on these properties of transformed cells? Many experiments were performed to determine the effects of the class of protease inhibitors which are derivatives of chloromethyl ketones on changes in cellular morphology (19,37-41), lectin agglutinability (38,40,42,43), hexose transport (19), surface LETS glycoprotein

expression (41), growth rate, and cell density (19,37-41,44-46) in both DNA and RNA virus-transformed cells and their untransformed counterparts. Unfortunately, chloromethyl ketone derivatives have been shown to inhibit protein synthesis in both untransformed and transformed cells (47,48). Thus the observations made in these experiments cannot be interpreted as simply the result of the inhibition of proteolytic enzymes. In fact, many of the observations can be mimicked by using cycloheximide, a known protein synthesis inhibitor, rather than the chloromethyl ketone derivatives (19,41,45).

Because of the difficulties in interpreting the results of studies using chloromethyl ketone protease inhibitors, only the results of studies using macromolecular protease inhibitors will be discussed.

Goldberg (43) found that transformed mouse, chick, and human cells became less agglutinable by wheat germ agglutinin when ovomucoid, a protease inhibitor from chicken egg white, was included in the growth medium. In contrast, Collard and Smets (42) found no reduction in agglutinability of transformed mouse cells grown in ovomucoid-containing medium.

Growing transformed hamster cells in the presence of ovomucoid, pancreatic trypsin inhibitor (PTI), or soybean trypsin inhibitor (STI), did not permit these transformed cells to express the LETS glycoprotein on their plasma membranes (41). However, as Hynes (41) points out, this does not rule out the involvement of proteases in the loss of LETS protein upon transformation.

Soybean trypsin inhibitor or ovomucoid had no effect on the high

levels of glucose transport observed in a transformed chick cell line (19). Other cell lines have not been investigated.

The growth properties of transformed 3T3 (38,42) and hamster kidney (38,46) cells were effected by ovomucoid and PTI. However, in some of these experiments it was shown that even though these cells stopped growing, they were not arrested in the G_1 phase of the cell cycle and thus this arrest did not represent a reversion to density-dependent inhibition of growth (42). Ovomucoid and PTI had no effect on another transformed hamster cell line (NIL8-HSV6) (41) or hamster tumor cells (37). Soybean trypsin inhibitor did inhibit the growth of hamster tumor cells (37), but had no effect on transformed 3T3 cells (38, Appendix I), transformed hamster kidney (38), or another transformed hamster cell line (NIL8-HSV6) (41).

In general, even when macromolecular protease inhibitors do have an effect on growth properties of transformed cells, the effects are small (a reduction in cell density of 20 to 30 percent (37,38)) and do not represent a reversion to density-dependent inhibition of growth (42).

The virus-transformed cell phenotypic characteristics in which protease inhibitors have been shown to have the greatest reversional effect are morphology, adhesiveness to the substratum, and proteolytic activity in the growth medium (assayed as fibrinolysis or caseinolysis). PTI and STI inhibit caseinolysis and fibrinolysis observed in transformed chick, mouse, and human cell cultures (49-52). STI also inhibits the plasminogen-independent fibrinolysis described by Chen and

Buchanan (53). Weber (19) has observed increased cellular adhesiveness in transformed chick cells grown in the presence of STI and that these cells tend to grow with a flatter, untransformed-like, morphology when STI is included in the growth medium. This change in morphology in the presence of PTI and STI has been observed by other laboratories for several different transformed cell lines (50-52, Appendix I).

The conclusions which can be drawn from these experiments with macromolecular inhibitors are somewhat limited, because even if proteases are responsible for certain characteristics of virus-transformed cells, the protease inhibitor chosen for the experiment may not inhibit the protease involved. Because the three inhibitors used in the experiments described all inhibit proteases with trypsin-like specificity, one may conclude that a trypsin-like enzyme which is inhibited by ovomucoid, STI, or PTI 1) may be involved in the increased agglutinability of transformed cells, 2) apparently is not involved in the loss of the LETS protein (at least in NIL8-HSV6 cells), 3) is not involved in the high levels of glucose transport (in transformed chick cells), 4) probably is not involved in the release from density-dependent inhibition of growth, and 5) is most certainly responsible for some of the increased proteolytic activity in transformed cell growth medium, the decrease in cellular adhesiveness, and some morphological changes (which hereafter will be referred to as protease dependent morphological change or PDMC).

Considerable evidence has been collected suggesting that virus-transformed cells have higher levels of proteolytic enzymes than their

untransformed counterparts (54-56), and that this enzymatic activity may play a role in changing the phenotype of transformed cells. The question remains, however, as to what proteolytic enzymes are responsible for this activity.

Reich's laboratory (39,50) established that fibroblast cultures from chick, hamster, mouse, and rat embryos, transformed by either oncogenic DNA or RNA viruses, produced an enzymatic activity capable of hydrolyzing ^{125}I-labeled fibrin, whereas untransformed embryo or 3T3 cultures did not possess this fibrinolytic activity. The fibrinolytic activity was shown to be produced by the interaction of two factors; a cell factor released into the medium of transformed cells, and a serum protein component. The serum factor was identified as plasminogen (57) and the cell factor as a serine protease which functions as a plasminogen activator (58). Through the interaction of these two factors, plasmin is generated in the transformed cell cultures and thus plasmin is thought to be responsible for the fibrinolytic activity observed in these cultures.

Even though the production of a high level of plasminogen activator in culture has become recognized as a distinct property of many transformed cells, some normal cells have been shown to produce this enzyme. Activated macrophages and cells from the lung, heart, vascular endothelium, and kidney produce plasminogen activators (59-63). Recently, it has been learned that cultured normal low-passage embryo fibroblasts from several different species elaborate increasing amounts of plasminogen activator as they approach confluence, and then lose this

plasminogen activator activity after reaching confluence (64,65). However, even when at their peak values, the plasminogen activator activities of the low-passage untransformed cells remains well below those of the corresponding virally or spontaneously transformed cells (64).

Plasminogen activators have been purified from transformed chick (58), mouse (62), hamster (66), rat breast carcinoma (67), human melanoma (52), human ovarian carcinoma (68), and human pancreatic carcinoma (69). All plasminogen activators of mammalian cells have a major component of 48,000 - 50,000 daltons, suggesting that these cell-derived plasminogen activators may be identical with urokinase, a plasminogen activator found in the urine (54,700 M.W.) (70). In fact, the plasminogen activator from human ovarian carcinoma has been shown to be immunologically identical with human urokinase (68).

Efforts to understand the increased proteolytic activity associated with transformed cells have concentrated on the plasminogen/plasmin system first defined by Reich's laboratory and elaborated by many others. However, the prothrombin/thrombin system has been considered by some investigators. Low concentrations of thrombin are known to produce changes in platelet plasma membranes (71,72). In addition, thrombin has been shown to be a mitogen (36) and to stimulate glucose uptake (20) in chick embryo cells. There is evidence that chick embryo cells can supply the thromboplastin activity (clotting factor III) required for prothrombin activation (72). Thrombin activity has yet to be demonstrated in cultures of untransformed and

virus-transformed cells grown in the presence of serum, however.

Regardless of the protease responsible for the phenotypic changes which occur upon transformation, if, in the simplest model, these changes are triggered by the hydrolysis of a specific cell surface protein, the plasma membrane is the most logical location for the activation of the protease so that it can function without being immediately inactivated by serum inhibitors. Indeed, in the case of the plasmin/plasminogen system there is some suggestive evidence that plasminogen can bind to the plasma membrane (50,51). Also, the plasminogen activator activity of cell homogenate fractions has been observed to increase after the addition of detergent (58,64,65), and the activator activity co-purifies with plasma membrane-rich fractions (73,74). These observations indicate that at least some portion of the total plasminogen activator activity is membrane bound and when considered in light of the plasminogen binding observations suggest the possibility of a specific cell surface-plasmin interaction.

To investigate how plasminogen does interact with cells in culture, Monty Krieger, Bob Stroud and I studied the binding and processing of ^{125}I-labeled dog plasminogen by untransformed and simian virus 40 (SV40) transformed Balb/c 3T3 cells. The experiments, which are described in detail in the appendices, were designed to answer three questions. First, is there a specific interaction of plasminogen with transformed cell surfaces (Appendix I)? Second, is the interaction of plasminogen with transformed cells related in some way to the protease-dependent morphological change (Appendix I)? Third, what

happens to plasminogen in the cell culture medium (Appendix II)?

As an initial step in answering the first of these questions, the overall binding of ^{125}I-plasminogen to the 3T3 cells was investigated. The amount of plasminogen associated with the 3T3 cells on a per-cell basis decreased exponentially over the three days of incubation, while the amount associated with the SV40 transformed 3T3 cells (SV3T3) was about the same on the third day as on the first. These overall binding data were not affected by inhibitors to the protease dependent morphological change, such as STI, and thus the binding of plasminogen is independent of PDMC.

The plasminogen which bound to the SV3T3 cells was completely degraded during the three days of incubation to macromolecules which were the same size as the large and small chains of active plasmin and to smaller fragments including 3-iodo-L-tyrosine (isolated by microstep exclusion chromatography; Appendix III). The plasminogen which bound to 3T3 cells was only partially degraded to 3-iodo-L-tyrosine, with intermediate conversion to plasmin-sized peptides. Through the use of STI, this processing of plasminogen was shown to be independent of PDMC.

To determine whether the processing of plasminogen by the cells was a cell surface phenomenon or an internal process, a sublethal cell-surface trypsinization assay was developed. The cell-associated plasminogen was trypsin-sensitive and thus assumed to be primarily bound to the surfaces of the 3T3 and SV3T3 cells. The macromolecular degradation products were not trypsin-sensitive and thus assumed to be inside of the cells.

Therefore, during the three-day incubation, 3T3 and SV3T3 cells bound, endocytosed, and degraded serum plasminogen. The overall rate of this processing was approximately twice as fast for SV3T3 cells as 3T3 cells. This binding and processing of plasminogen does not appear to be a specific plasma membrane/plasmin interaction, however. These data do not allow differentiation between plasminogen being transported into the cell by pinocytosis or site-specific binding and uptake. Regardless of which of these processes are responsible for the internalization of plasminogen, the overall binding and processing of plasminogen is known to be independent of PDMC.

As to what happens to plasminogen in the incubation medium, essentially none of the plasminogen incubated with the 3T3 cells was activated to plasmin, indicating that these cells do not elaborate plasminogen activator into the growth medium. Some of the plasminogen which was incubated with the SV3T3 cells was activated to plasmin and subsequently complexed with a serum inhibitor of 47,000 MW. This inhibitor is covalent, forming a hydroxylamine-dissociable bond and is thought to be similar to the antiplasmin which has been described for human plasma (75-77).

The question then arose whether all of the plasmin generated in the SV3T3 incubation medium was inhibited. Through the use of a model system of urokinase _in situ_ activation of plasminogen designed to mimic the levels of activation observed in a 48-hour incubation of plasminogen with SV3T3 cells, it was found that some plasmin incorporated ^{32}P-labeled diisopropylfluorophosphate, a covalent inhibitor of serine

proteases. The implication of the result is that active plasmin does exist in growth medium and thus may be responsible for the protease dependent morphological change and other phenotypic changes which occur upon virus transformation.

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APPENDIX I

THE BINDING AND PROCESSING OF DOG PLASMINOGEN BY BALB/c 3T3 and SV3T3 CELLS*

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SUMMARY

Plasminogen binding to Balb/c 3T3 and SV3T3 cells was studied using ^{125}I-labeled dog plasminogen. The binding appears to be independent of the protease-dependent morphological change (PDMC)[1] which is exhibited by many transformed cells. The ^{125}I-plasminogen which bound to the SV3T3 cells was completely degraded during three days of incubation to macromolecules which were the same size as the large and small chains of active plasmin, and to smaller fragments including 3-iodo-L-tyrosine. The plasminogen which bound to 3T3 cells was only partially degraded to 3-iodo-L-tyrosine with intermediate conversion to plasmin-size peptides. The SV3T3, and to a somewhat lesser extent, the 3T3 cells, accumulated 3-iodo-L-tyrosine during the course of the incubation. In the incubation media, only a small fraction of plasminogen was degraded to the level of individual amino acids (5%).

The results of a sublethal cell-surface trypsinization assay suggest that the cell-associated plasminogen was primarily bound to the surfaces of the 3T3 and SV3T3 cells while the macromolecular degradation products were inside the cells. There were significant differences in the rates of macromolecular degradation exhibited by the 3T3 and SV3T3 cells which presumably reflect differences in endocytosis or lysosomal hydrolysis, or both.

INTRODUCTION

The enhanced proteolytic activity of cells transformed by oncogenic DNA and RNA viruses, or chemical carcinogens, can be important in mediating certain morphological changes characteristic of transformation (1,2). Several neoplastic and virally transformed cells have been shown to synthesize an enzyme which can activate serum plasminogen to plasmin (3-10). The increased proteolytic activity of transformed cells and the protease-dependent morphological change (PDMC) may be due to plasminogen activation (11,12). Cells which have undergone PDMC are spherical, tend to clump, and retract from the substratum (1,2). When simian virus 40 transformed hamster embryo fibroblasts (SVHEF) are grown in media supplemented with plasminogen-depleted serum (13), or in serum containing high concentrations of plasmin inhibitors (2), the morphological changes do not occur (12).

Ossowski et al. (13) reported that SVHEF cells bound more ^{125}I-labeled plasminogen (or possibly plasmin) than their normal counterparts, and that soybean-trypsin inhibitor (STI) decreased binding to the transformed cells. These binding data and reports of proteolytic activity associated with the plasma membranes of transformed cells (14) suggested the possibility of a specific cell surface-plasmin interaction. We have studied the binding and processing of ^{125}I-labeled dog plasminogen by untransformed and SV40-transformed Balb/c 3T3 cells and its relationship to the PDMC exhibited by the transformed cells.

used for UV and visible spectrophotometry. The enzymatic activity of the plasminogen (20) activated with Varidase (Lederle, Lot 406-334) or urokinase (Calbiochem, Lot 501639), 2330 plough units/vial, was unaffected by the iodination.

To determine the appropriate conditions for urokinase activation, 100 µl of iodinated plasminogen (0.5 mg/ml in PBS) were incubated with 70 µl of urokinase (1 vial/0.25 ml Tris buffer) at room temperature. At various times the extent of plasminogen activation was assayed spectrophotometrically by following the hydrolysis of N_{α}-carbobenzoxy-L-lysine-p-nitrophenyl ester (Cyclo Chemicals Lot D-1308) (20), and by electrophoretic determination of the molecular weight distribution of the iodinated species in the reaction mixture.

Gel Electrophoresis

Radiolabeled samples were electrophoresed in 5 mm diameter glass tubes on a 9 cm 10% polyacrylamide separating gel allowed to polymerize for three to four days, overlayered with a 1 cm 3% stacking gel prepared according to Laemmli (21). Samples were reduced and denatured in 3% SDS-BME-Tris buffer by immersion in boiling water for three minutes immediately before electrophoresis. The volume of the sample loaded on the gels ranged from 10-200 µl. When the samples were radioactive, they contained 5,000 - 30,000 cpm. The gels were electrophoresed at 2 mA per tube for approximately five hours. The schlieren line due to the stacking buffer system was not allowed to run out of

the gel during electrophoresis. The gels were frozen immediately after electrophoresis, sliced into 1-mm long pieces, and counted. For all electrophoretograms presented, the electrophoretic mobility scale (R_f) is based on the mobility of the bromophenol blue marker dye and the radioactivity in the gel. The smooth curves in the figures pass through all of the observed data (80-90 slices).

The dependence of the R_f on molecular weight was determined from Coomassie blue-stained gels (staining solution: 0.25% Coomassie blue, 25% propanol, 10% acetic acid; destaining solution: 10% acetic acid) using myosin (210,000 daltons), phosphorylase A (92,000 daltons), bovine serum albumin (68,000 daltons), catalase (57,000 dalton subunit), aldolase (40,000 dalton subunit), and myoglobin (17,200 daltons) as molecular weight standards. Samples of these proteins were provided by Dr. Richard Vandlen.

The molecular weights of dog plasminogen and the light chain of dog plasmin (see Results) were found to be 93,000 ±6,500 and 25,000 ±800, respectively. The errors represent the standard deviations determined from nine (plasminogen) and eleven (plasmin) independent electrophoretograms. These molecular weights were used as internal standards to calibrate the other electrophoretograms because the extent of polyacrylamide polymerization, and thus the electrophoretic mobilities of the samples, were not identical for all gel preparations.

Binding Studies

The Balb/c 3T3 and Balb/c SV3T3 cells used were obtained from Dr. G. Todaro and were maintained in an incubator (37°C, 5% CO_2 atmosphere) in Dulbecco's modified Eagle's medium (Gibco) with 4.5 times the glucose concentration and supplemented with 10% fetal calf serum (10% FCS). The cells were periodically checked and found to be free of mycoplasmal infection (22,23). All cell incubations were carried out in a 37°C incubator (5% CO_2 atmosphere), and all other procedures were conducted at room temperature unless otherwise noted.

To determine the extent and the time course of plasminogen binding to the cells, the cells were plated at 4 x 10^5 cells/60 mm petri dish (Nunclon) in 5 ml of 10% FCS-supplemented medium. After one day, the medium was removed and the cultures were washed twice with isotonic Tris buffer. Fresh medium supplemented with either 10% dog serum (Gibco) (10% DS) or 2% dog serum (2% DS), and ^{125}I-labeled dog plasminogen in PBS was added. The final concentration of ^{125}I-plasminogen was ∿100 nM. The plasminogen concentration in 5% dog serum-supplemented medium has been estimated to be 34 μg/ml (12); therefore, approximately 12% of all plasminogen in the incubation medium was ^{125}I-labeled.

After incubation periods of 24, 48, and 72 hours, the cell morphology was scored to determine the extent of PDMC according to the criteria of Ossowski et al. (12), the incubation medium was removed, and the cells were detached from the petri dish using a rubber policeman. The cells were washed in 20 ml Tris buffer suspensions and

pelleted (2500 rpm for five minutes) between each wash until they were free of unbound radiolabeled material ("Off-Plate" washing procedure). Ten to fifteen wash cycles were normally required to bring the supernatant counts down to background levels. After resuspension in a one-to-one mixture of 10% FCS-supplemented medium and a 1% solution of trypsin (Worthington Biochemical Corp. Lot TRL35M960) in PBS, the number of cells was determined using hemocytometers (at least four independent determinations), and the radioactivity in the suspension was measured. The results of the binding studies will be presented as equivalent molecules of plasminogen bound per cell. These data are calculated from the specific activity of the plasminogen and the amount of cell-associated radioactivity. The standard deviation in cell-associated radioactivity for any one experiment determined from replicate samples was about 20% of the total binding. Nine binding experiments were conducted, each one involving two to eight replicate dishes per cell type per day.

Similar binding studies were also conducted with SVHEF cells kindly provided by Dr. D. Rifkin. The SVHEF cells were maintained under conditions identical to those for the Balb/c cells. For the binding studies, the SVHEF cells were incubated in 2% DS-supplemented medium and were washed in one of two ways. In one set of experiments, the cells were washed free of unbound radiolabeled material while attached to the substratum ("On-Plate" washing procedure). The cells were then released from the plate by trypsinization, suspended in the trypsin solution and counted. In other experiments, the Off-Plate

wash was used.

Because a change in plasminogen binding to the SV3T3 and SVHEF cells might accompany PDMC on the third day of the binding experiments, a number of PDMC inhibitors and an accelerator of PDMC were included in the incubation media to assess the relationship between plasminogen binding and PDMC. STI (1,2) and PTI (pancreatic-trypsin inhibitor) can inhibit the PDMC expressed by SV3T3 and SVHEF cells grown in DS-supplemented medium. PDMC is also inhibited if the cells are incubated in FCS rather than DS-supplemented medium (2). In order to inhibit the PDMC during plasminogen binding experiments, STI (Worthington Biochemical Corp. Lot 54J358) or PTI (Sigma Lot 81C-8200) was added to the DS-supplemented incubation medium at t = 0, or 10% FCS was substituted for DS in the medium. The concentrations of STI (14.6 μM for SV3T3 cells) and PTI (6.7 μM for SV3T3 and 14 μM for SVHEF cells) used were the minimum concentrations necessary to inhibit PDMC and were determined by dose-response studies. The commercial STI was chromatographed on Sephadex G-75 at 4°C to remove contaminants. Varidase, which contains the plasminogen activator streptokinase, was used to accelerate the time course of PDMC by one day during a plasminogen binding experiment. The Varidase concentration required to do this (83.3 streptokinase units per dish) was determined by dose-response analysis. If a higher Varidase concentration was used, the cells were reduced to debris within 24 hours.

The binding of other ^{125}I-labeled proteins to the 3T3 and SV3T3 cells was measured to compare with the binding of plasminogen. The

proteins, their specific activities, and their concentrations in the incubation media were: PTI (1.5×10^8 cpm/mg, 0.61 μM); STI (1.2×10^7 cpm/mg, 14.5 μM); avidin-biotin complex (9.1×10^7 cpm/mg, 0.16 μM); ovalbumin (4.4×10^7 cpm/mg, 0.21 μM, two-times recrystallized, Worthington Biochemical Corp. Lot 0435A953). The avidin-biotin complex was made by mixing an excess of biotin (Sigma Lot 94C-0187) with avidin (Sigma Lot 104C-0117) and purifying the iodinated complex on a Sephadex G-25 column at 4°C. The experimental procedure for the binding assay was identical to that used for plasminogen. The cells were growing logarithmically throughout the duration of all binding studies.

Characterization of Cell-Associated ^{125}I-Labeled Material

The molecular weight distributions of the cell-bound iodinated molecules were analyzed by gel electrophoresis. After the radiolabeled cells were washed free of unbound radioactivity, they were dissolved in 3% SDS-BME-Tris buffer, and the macromolecules were denatured and reduced. To decrease the viscosity of the samples, the solutions were then vigorously passed through a Pasteur pipette. The cell solutions were then electrophoresed.

To further characterize the very low molecular weight iodinated molecules found associated with the SV3T3 cells, the LMW was isolated using micro-step-exclusion chromatography (24) and analyzed by ascending paper chromatography (25).

Characterization of ^{125}I-Labeled Material in the Incubation Media

The molecular weight distributions of iodinated molecules in the incubation media were analyzed by denaturing gel electrophoresis and gel filtration of Sephadex G-150 (90 cm x 4.9 cm^2, 0.1 M ammonium bicarbonate buffer, pH 7.9). Samples of the incubation media were mixed in the 3% SDS-BME-Tris buffer and reduced and denatured. In one experiment the incubation media were also denatured in 3% SDS-BME-Tris buffer lacking β-mercaptoethanol so that the unreduced electrophoretic pattern could be detected.

The iodinated low molecular weight molecules in the incubation media were isolated using Bio-Gel P-2 micro-step-exclusion chromatography (24). Twenty-five µl samples of the incubation media were loaded on micro-step-exclusion chromatography columns which were equilibrated with PBS, and the LMW molecules were eluted in 50 and 100 µl steps. The LMW material was eluted after passing 350 µl of PBS through the columns. The LMW was then subjected to descending paper chromatography (n-butanol:acetic acid:H_2O); 4:1:2; v/v/v, (25)) on Whatman No. 3MM paper. Standard samples of 3-iodo-L-tyrosine (MIT, Aldrich Lot 082837) and 3,5-diiodo-L-tyrosine (Aldrich Lot 071537) were co-chromatographed. The locations of the MIT and diiodo-L-tyrosine were determined by ninhydrin spray staining (0.2% v/v) and the paper was cut and the pieces counted in a gamma spectrometer to determine the mobilities of the iodinated species. The mobilitiy of I^- was determined in a separate experiment in which the I^- was visualized by staining with I_2 vapor.

To compare the results from the above procedure with those obtained by the more usual trichloroacetic acid (TCA) precipitation assay, the acid solubility of the radiolabeled molecules in the incubation media was measured. One ml of cold (0°C) 10% TCA was added to 25 µl of incubation medium and allowed to stand at 0°C for 90 minutes. The sample was then centrifuged at 10,000 g and 4°C for 30 minutes, and the amounts of radioactivity in the pellet and supernatant were determined.

Trypsinization Assay

A sublethal cell-surface trypsinization assay was developed in an attempt to determine what fraction of the cell-associated radioactive material was cell-surface bound, and how much was internalized. 3T3 and SV3T3 cells washed free of unbound radioactivity were resuspended in a one-to-one solution of 10% FCS-supplemented medium: 1% trypsin (w/v in PBS). At various time intervals, aliquots were assayed for release of cell-associated radioactivity by pelleting the cells at 4°C for 30 minutes at 7000 g and determining the relative amounts of radioactivity in the supernatant and in the pellet. Cell viability under these conditions was checked by Trypan Blue exclusion (26) and by measuring the relative plating efficiency and growth of the cells in 10% FCS-supplemented medium after the trypsinization treatment. A twenty-minute trypsinization removed 90% of the radiolabeled material which could be removed without affecting cell viability (see Results).

To determine the effects of the trypsinization, cells were washed thoroughly after incubation with ^{125}I-plasminogen and trypsinized for twenty minutes. The mixture was then diluted four to twenty times with TD buffer, and the cells were pelleted, dissolved in the 3% SDS-BME-Tris buffer, prepared for electrophoresis, and electrophoresed, as described above. The TD buffer diluted supernatant was counted to find the amount of radioactivity released by the trypsin treatment. In one of the three trypsinization experiments that were performed, twenty-minute control incubations in which trypsin was omitted were conducted at 0°C and at room temperature (approximately 22°C).

RESULTS

Plasminogen Activation and the Molecular Weights of Plasminogen and the Subunits of Plasmin

The plasminogens of several different species have been shown to be single polypeptide chains with molecular weights of about 90,000 (27,28). During urokinase activation, two peptide bonds in plasminogen are cleaved (29). One cleavage yields a two-chained, disulfide-linked molecule with the smaller (trypsin-like) active-site-containing subunit (25,000 daltons) derived from the carboxyl terminus of plasminogen. The second cleavage which occurs near the N-terminus releases a 6,000-8,000 dalton peptide. In the presence of macromolecular trypsin

inhibitors (30,31), the second cleavage which is due to hydrolysis by plasmin itself does not occur.

Urokinase treatment of ^{125}I-labeled dog plasminogen produced a rapid increase in plasmin activity which began to plateau after five minutes of incubation; however, electrophoretograms showed that there were small, yet significant amounts of plasminogen after 30 minutes of incubation. Essentially, no plasminogen remained after 60 minutes of incubation (Figure 1), and there was no evidence of autolysis. Therefore, an hour incubation was adopted as the standard time for urokinase activations.

An electrophoretogram of the urokinase-plasminogen activation mixture reduced and denatured after one minute of incubation shows five distinct components (Figure 1): plasminogen (MW 93,800); B_1, plasmin heavy chain (MW 74,100); B_2, plasmin heavy chain derived from B_1 (MW 66,500); C, plasmin light chain (MW 25,000); and a low molecular weight component, L, with an electrophoretic mobility (R_f = 0.98) which is slightly less than the bromophenol blue marker dye. The B_1 chain does not appear in electrophoretograms of the reaction mixture taken after 16, 30, and 60 minutes of incubation. Electrophoresis of samples which have not been reduced shows that the B and C proteins are cross-linked by at least one disulfide bond. We conclude that the C-chain is the active site containing trypsin-like small subunits of dog plasmin, that the B_1 and B_2 chains are the large subunits before and after the N-terminal cleavage which accompanies urokinase activation, and that a small peptide, L, is the N-terminal peptide with a molecular

weight of approximately 7,600 daltons (the difference in molecular weight between the B_1 and B_2 chains). The early, transient appearance of the B_1 chain is consistent with the suggestion that plasmin is responsible for the cleavage of the 8,000 MW N-terminal peptide, $B_1 \rightarrow B_2 + L$ (30,31).

In addition to providing the molecular weights of the plasmin subunits, the electrophoretograms of activation mixtures also show the relative extent of ^{125}I incorporation into the subunits. From the electrophoretogram of the one-minute incubation mixture, the ratios of specific activities (cpm/mole) are calculated to be $B_1:B_2:L:C = 0.97:0.71:0.26:1.0$. The ratio of $B_2:C$, determined from ten electrophoretograms of three independent iodinations of plasminogen, is $0.70 \pm 0.09:1.0$, which indicates that the ^{125}I-labeling pattern is reasonably reproducible, and that the small subunit contains more accessible tyrosines than does the large subunit.

Protease-Dependent Morphological Change

Dog serum is an "activating serum" (2) for SV3T3 and SVHEF cells because the cells undergo PDMC when they are grown in dog serum-supplemented media. We found that the SVHEF cells exhibited a dramatic PDMC between the second and third days of incubation in either 10% DS or 2% DS-supplemented medium. A less marked PDMC occurred in the SV3T3 cultures between 24 and 48 hours after transfer to DS-supplemented medium. A more obvious PDMC occurred between the second and

third days; however, the PDMC was not as dramatic with the SV3T3 cells as with the SVHEF cells. 14.6 μM STI or 6.7 μM PTI were required to completely inhibit PDMC expressed by the SV3T3 cells (Figure 2). Fifteen μM PTI were required to inhibit the PDMC occurring with the SVHEF cells, and 0.6 μM PTI was sufficient to inhibit the PDMC manifested by a line of mycoplasma-infected Swiss SV3T3 cells. Swiss SV3T3 and Swiss 3T3 cells were provided by Dr. J. Vinograd and maintained in culture as described for the Balb/c cells. These results are consistent with the findings that PDMC depends upon serum protease activation by a cell synthesized activator (1,2). The different cell lines may require different concentrations of inhibitors to prevent PDMC because the concentration of protease required to elicit PDMC or the rates of protease activation depend on the cell type.

Fetal calf serum is a "nonactivating serum" (2) for SV3T3 and SVHEF cells, and no PDMC was observed when the cells were grown in 10% FCS. In contrast to previous reports of the effects of trypsin inhibitors on cell growth (32), we found no differences in the doubling times for Balb/c SV3T3 (16.3 hours) or SVHEF (14 hours) cells grown in media which included either 2% DS, 10% DS, or 10% FCS alone, or 2% or 10% DS supplemented with STI or PTI. Varidase, which contains the plasminogen activator streptokinase, could accelerate the onset of PDMC in Balb/c SV3T3 cells; however, the concentrations of Varidase necessary to produce a one-day shift in the PDMC time course (83.3 streptokinase units/dish) completely inhibited cell growth.

The untransformed Balb/c and Swiss 3T3 cells do not exhibit a PDMC; however, trypsin inhibitors did delay the detachment of the cells from the petri dishes when the cells were allowed to grow for extended periods (five to eight days). This suggests that a trypsin-like serine protease may be involved in the detachment process for untransformed cells.

Plasminogen Binding

After incubation for one, two, or three days in ^{125}I-plasminogen and 10% DS-supplemented medium, the Balb/c 3T3 and SV3T3 cells were removed from the petri dishes by scraping, thoroughly washed using the Off-Plate procedure, and counted to determine their specific activity. The results from nine separate experiments expressed in plasminogen molecules per cell for all experiments are shown in Figure 3. With few exceptions, the data for the SV3T3 cells fall into a shallow V-shaped pattern with the greatest spread in the data at 24 hours. There is much greater variability in the 3T3 binding data, even though the standard deviation in binding for replicate dishes from any one experiment was about the same for both cell types. The plasminogen binding per 3T3 cell decreased exponentially over the three-day incubation period. Again, the greatest spread in the data occurred at 24 hours. During the incubations, the SV3T3 and 3T3 cells doubled in number every 16.3 and 16.5 hours, respectively. Binding curves for SVHEF cells (Figure 4) show that plasminogen binding increased

throughout the three-day incubation with the largest increase occurring between the second and third days.

The concentration dependence of plasminogen binding was not determined, however the extent of binding (10^4 - 10^5 molecules/cell), determined using the Off-Plate procedure, was not remarkably different from that of other proteins (Table I).

Inhibitors of PDMC (STI, PTI, and FCS) were added to the incubation media in several binding experiments in order to assess the relationship between PDMC and plasminogen binding. The ratios of the extent of plasminogen binding in the presence of inhibitors to that observed without the inhibitors are listed in Table II. The values in the table are the averages of the ratios observed on each of the three days of the experiments. The effect of PTI on plasminogen binding was found to depend on when the inhibitor was added to the incubation media, and the cell-washing procedure. The amount of PTI added was sufficient to inhibit PDMC (see above). A "pulse" of PTI added two hours before the cells were assayed for plasminogen binding had no effect on the binding regardless of the washing procedure. When the PTI was added at t = 0 and left in the medium throughout, it had little if any statistically significant effect on the plasminogen binding to the Balb/c 3T3, Balb/c SV3T3 or SVHEF cells washed using the Off-Plate procedure. When the On-Plate wash was used, PTI added to the incubation media of Balb/c SV3T3 or SVHEF cells at t = 0 reduced the apparent plasminogen binding by about 50%. Ossowski et al. (13) reported that STI blocked the uptake of ^{125}I-labeled plasminogen by SVHEF cells when

the binding was assayed using the On-Plate wash procedure. Their experiments differed from those reported here in that the dog serum used to supplement the incubation medium was subjected to affinity chromatography and dialysis to remove endogenous plasminogen, and the time course of binding was followed for 24 hours rather than 72 hours.

STI had essentially no effect on the plasminogen binding to Balb/c SV3T3 cells and apparently induced a slight increase in binding to Balb/c 3T3 cells (Table II). In these experiments, STI was added at t = 0 and the cells were washed with the Off-Plate technique. The effects of FCS on plasminogen binding are difficult to assess because of the substantial variability in the results (Table II).

The differences between the Off-Plate and the On-Plate assay results may be due to two problems associated with the On-Plate technique:

1) The On-Plate wash assay may monitor dish-associated as well as cell-associated radioactivity. This possibility is suggested by the following controls: SV3T3 cells which were incubated for three days in ^{125}I-plasminogen and 10% DS-supplemented medium were washed while attached to the petri dish, then removed from the dish with a rubber policeman. The cell-free plates were extensively washed with Tris buffer and then trypsinized. The trypsinization solution contained ^{125}I-radioactivity. In addition, the measure of "plasminogen binding" was higher when SVHEF cells were washed on the plates than when the Off-Plate wash was used.

2) Another problem with the On-Plate wash is that free-floating or loosely bound cells are washed away, thus possibly biasing the cell population with a large percentage of firmly attached cells. This selection might be particularly significant after PDMC occurs when many cells have retracted from the petri dish. In contrast, the entire cell population is assayed when the Off-Plate procedure is used.

Characterization of the Cell-Associated Radiolabeled Molecules

^{125}I-radiolabeled molecules associated with the cells were analyzed by reducing and denaturing polyacrylamide gel electrophoresis, Bio-Gel P-2 micro-step-exclusion chromatography and paper chromatography. Electrophoretograms of 3T3 and SV3T3 cells revealed the same five predominant species in varying relative amounts (Figure 5). These include plasminogen (93,800 MW) and two peaks of lower molecular weight designated B_1 (74,600 MW) and C (25,000 MW). There is also a peak of very low electrophoretic mobility (HMW), and a broad peak of low molecular weight. The molecular weight of the B_1 protein is very similar to that of the large fragment B_1 of plasmin, and the molecular weight of the C protein is identical to that of the C chain of plasmin, suggesting that these peaks are due to activation products of plasminogen. The HMW must be due either to contaminants in the iodinated plasminogen or to an artifact of the electrophoretic system.

The broad LMW peak appears to be mostly monoiodotyrosine based on the following single experiment. 55% of the total radioactivity in

Balb/c SV3T3 cells solubilized after 72 hours of incubation was associated with molecules small enough to be retained by a Bio-Gel P-2 (<1800 MW) micro-chromatographic column (24). This material co-migrated with monoiodotyrosine during ascending paper chromatography. Since approximately 60% of the total radioactivity in these Balb/c SV3T3 cells migrated as LMW during gel electrophoresis, almost all of the cell-associated LMW in this case was MIT. The electrophoretograms for the Balb/c 3T3 cells for a typical experiment (Figure 5) show that plasminogen is the major iodinated cellular component. The relative amount of plasminogen diminished with increasing time of incubation while the B', C' and LMW peaks developed. The relative intensities of these peaks expressed as a percentage of total macromolecules (>7000 daltons) and averaged over four independent experiments are depicted in Figure 6.

The data in Figure 5 show that there is a more pronounced change in the molecular weight distributions of the SV3T3 cell-associated ^{125}I-labeled macromolecules over the course of the incubation. Most strikingly, there was no plasminogen associated with the SV3T3 cells after three days of incubation even though plasminogen was present in the medium ($\sim$55 nM). All of the SV3T3 cell-associated plasminogen appears to have been completely degraded. This apparent degradation was accompanied by an increase in the relative amounts of C' protein and a minor increase in the B' protein when expressed as a fraction of all macromolecules.

Concomitant with these changes in the relative amounts of these macromolecular species, there was an overall increase in the amount of LMW and an overall decrease in the total amount of cell-associated macromolecules (Figure 7). Therefore it appears that the Balb/c SV3T3 associated plasminogen was degraded to low molecular weight fragments as small as monoiodotyrosine, via B' and C' intermediates, and that the rates of formation and/or degradation of the B' and C' proteins were different. The degradation of plasminogen bound by SV3T3 cells was much faster than for 3T3 cells. By the third day of incubation, 75% of the SV3T3 cell-associated radioactivity was in the form of LMW while this was only 35% for the 3T3 associated radioactivity (Figure 7). At the same time there was almost no plasminogen remaining associated with the SV3T3 cells, whereas 45% of all 3T3 associated material was in the form of undigested plasminogen. Unlike the case with the SV3T3 cells, the rates of formation and/or degradation of the B and C proteins associated with the 3T3 cells appear to be identical.

The addition of STI to the incubation media of both 3T3 and SV3T3 cells at a concentration sufficient to inhibit PDMC had essentially no effect on the molecular weight distribution of the cell-associated ^{125}I-labeled molecules (Figure 8). Therefore, it is unlikely that an STI-inhibitable protease--for example, plasmin--in the media is responsible for the degradation of the cell-associated plasminogen. The STI results also show that the cell-associated plasminogen degradation is independent of PDMC.

Localization of Cell-Associated Radiolabeled Macromolecules

To localize the cell-bound ^{125}I-labeled protein, cells were trypsinized with the goal of removing cell-surface proteins without affecting molecules which had been internalized. The molecular weight distribution of the remaining cell-associated radiolabeled molecules was determined by electrophoresis.

The time course of ^{125}I release from labeled cells showed an exponential release of radioactivity which plateaued when approximately 50-80% of the cell-bound radioactivity was released by the trypsinization (Figure 9). The kinetics of release were similar for the Balb/c 3T3 and SV3T3 cells. Half of the counts which could be released by the trypsin treatment were released after six minutes, and the plateau was reached by approximately 50 minutes of trypsinization. If the period of trypsinization was extended beyond 100 minutes, there was an additional release of radioactivity. However, cell viability assayed by relative plating efficiency began to fall after 60 minutes of trypsinization, so the increased release of radioactivity after prolonged trypsinization was probably caused by cell damage and release of internalized radioactivity. There was no indication of cell damage assayed by Trypan Blue exclusion for up to 120 minutes of trypsinization. A twenty-minute standard trypsinization incubation time was used because approximately 90% of the trypsinizable counts were released after this period without loss of cell viability.

To assess the effect of the twenty-minute trypsinization, samples of cells taken before the trypsin incubation, after incubation with trypsin at 22°C, and after control incubations without trypsin at 22°C and at 0°C, were electrophoresed. The electrophoretograms show that all of the ^{125}I-plasminogen and the HMW bound to the 3T3 cells is removed by the tryptic hydrolysis (Figure 10). The degradation products remaining on the cells after trypsinization were similar in molecular weight to the B' and C' proteins and LMW. The control incubations of 3T3 cells at both 0°C (where endocytosis is known to be inhibited) and room temperature (22°C) did not alter the electrophoretic patterns seen before trypsinization. Thus, it is unlikely that 3T3 cells themselves degrade plasminogen during the trypsin treatment. Since the controls without trypsin show no detectable change in the electrophoretograms (Figure 10), the clear indication is that all of the plasminogen is located on the cell surface.

Similarly, the degradation of Balb/c SV3T3 cell-associated plasminogen by trypsin indicates that plasminogen is cell-surface bound. After 48 or 72-hour incuation there was little or no cell-bound plasminogen, and the only effects of trypsin treatment were diminution in the amounts of cell-bound B' and LMW. The identical effects, however, are also seen in the 0°C and room temperature controls where trypsin is left out, which shows that these changes are not a result of tryptic hydrolysis. Thus, the indication again is that all plasminogen was bound on the cell surface. Degradation of plasminogen probably occurred inside the cell in the lysosomes.

The effect of the cell-surface trypsin treatment was also monitored by determining the amount of radioactivity released into the medium (Table III). The radioactivity released included material whose release was independent of tryptic hydrolysis which can be determined from the room temperature control and material which was specifically released from the cell surfaces by trypsin. The amount of material released specifically by trypsin (heavy line, Figure 11) was calculated by subtracting the amount of material released during the room temperature control from the total amount released in the presence of trypsin (light line, Figure 11). The amount of SV3T3 cell-bound material which was released specifically by trypsin decreased with the time of incubation in a fashion similar to that seen for the SV3T3 cell-associated plasminogen (Figure 6). This is consistent with the electrophoretic data which suggest that the primary effect of trypsinization was the digestion of plasminogen on the cell surface. When there was essentially no plasminogen on the cells (SV3T3, t = 72), there was essentially no trypsin-specific release of radioactivity.

The release of radiolabeled material which occurred in the absence of trypsin was substantial (Table III) and showed a small (highly variable) temperature dependence. There was a slightly greater release at room temperature than at 0 °C where exocytosis should be inhibited; however, the electrophoretograms of cells subjected to the 0°C and room temperature incubations were nearly identical.

It is not possible to make simple generalizations about the kinds of molecules released during the control incubations. From the

electrophoretograms it appears that LMW was the principal but not the sole constituent released by the SV3T3 cells. There is almost no difference in the electrophoretic patterns of the 3T3 cells before and after the control incubations which suggest that all of the cell-surface bound radiolabeled species (primarily HMW and plasminogen) were released in equal proportions.

Cellular Turnover

The rate of appearance of MIT in the incubation media is a frequently employed measure of cellular digestion of iodinated proteins (41-44). To determine the amount of MIT in the incubation media of one experiment, the ^{125}I-labeled LMW components of the media were separated from the macromolecules by micro-step-exclusion chromatography using Bio-Gel P-2 (24), and then subjected to paper chromatography. Four distinct radioactive species were resolved in the chromtograms of all of the LMW samples (Figure 12): one co-migrated with MIT and one co-migrated with I^-. Of the other two components, one migrated near the solvent front and the other remained at the origin. These components have not been further characterized. The amounts of separated LMW components in the media versus time of incubation are listed in Table IV.

The preferential adsorption of MIT or I^- or both to the Bio-Gel P-2 chromatographic column could introduce artifacts in these results. However, a TCA precipitation of the 72-hour SV3T3 cell incubation

medium showed that 4.8% of the radioactivity in the medium was acid soluble. This is sufficiently close to the value of the amount of MIT plus I^- determined by micro-step-exclusion chromatography (5%) to suggest that preferential adsorption is an unlikely source of error.

If we assume that the rate of plasminogen degradation to LMW components followed by exocytosis for a given cell type is $\underline{k}$, then the total amount of LMW at time $\underline{t}$, due to processing by the cells, will be given by

$$(LMW)_t - (LMW)_0 = \int_0^t k\ No\ 2^{t/t_D}\ dt,$$

where No is the initial number of cells at time t = 0 (No equals 3×10^5 for the SV3T3 cells and 1×10^5 for the 3T3 cells), and t_D is the doubling time for the logarithmically growing cells (t_D equals 16.3 hours for the SV3T3 cells and 16.5 hours for the 3T3 cells). Thus,

$$(LMW)_t - (LMW)_0 = k\ No\ \frac{t_D}{\ln 2}\ (2^{t/t_D} - 1).$$

The constant background level, $(LMW)_0$, accounts for the initial amount of LMW in the supplemented medium (and for possible perturbations at the time of plating (t = 0)). Using the data in Table IV in which concentrations are expressed as the percent of the total amount of

radioactivity in the medium, the value of $\underline{k}$, the rate of formation of LMW for SV3T3 cells, is calculated to be $3.0 \pm 0.5 \times 10^{-8}$, and $(LMW)_0$ is 4.1 ± 0.4. The rate for 3T3 cells is $1.8 \pm 3.2 \times 10^{-8}$, and $(LMW)_0$ is 4.3 ± 0.9. Therefore, the overall turnover rate for SV3T3 cells is about two times greater than for normal 3T3 cells, even though the doubling time for SV3T3 cells is almost equal to that of 3T3 cells. If the rates are computed for MIT alone, the rate for SV3T3 cells is $1.5 \pm 0.002 \times 10^{-8}$, and for 3T3 cells about 2.5 times slower, $0.6 \pm 3.2 \times 10^{-8}$, with $(MIT)_0$ equal to 0.33 ± 0.0002 and 0.5 ± 0.9, respectively.

Characterization of Radiolabeled Protein in the Incubation Media

The ^{125}I-labeled molecules in the incubation media were analyzed by electrophoresis and gel filtration. The ^{125}I-plasminogen added to the 3T3 cell incubation media was essentially unaffected by the incubation.

Reducing and nonreducing electrophoresis of the SV3T3 incubation media showed that an increasing proportion of the plasminogen (increasing to 45% after three days of incubation) is degraded. The degradation generated smaller polypeptides of molecular weights 74,000, 12,000 and 10,000 (see Figure 13), which are linked together by disulfide bonds. There was essentially no 25,000 molecular weight species in the Balb/c SV3T3 incubation media. It is likely that some of the plasminogen in the medium was activated to plasmin and that the

observed peptides were derived from the two subunits of plasmin. If this was the case, most of the active site containing C subunit of plasmin must have been modified either by degradation to 10,000 and 12,000 molecular weight species or by covalently binding to a 50,000 molecular weight serum component forming a 74,000 molecular weight complex. This serum component may be a plasmin inhibitor, and may be similar to the 50,000 molecular weight inhibitor described by Collen (45) and Müllertz and Clemmensen (46).

Chromatography on Sephadex G-150 showed that all the plasminogen hydrolysis products were bound together in a specific complex which has a molecular weight greater than 210,000. Further analysis of this complex is described elsewhere (47).

DISCUSSION

Overall Pathway

During the course of a three-day incubation in 10% dog serum-supplemented medium, Balb/c 3T3 and Balb/c SV3T3 cells bound, endocytosed, and degraded serum plasminogen the serine protease precursor which has been implicated in the proteolytic modification of transformed cell morphology. The observations which support this overall pathway are:

1) Intact ^{125}I-plasminogen and intermediate products of plasminogen degradation (B' and C' protein, MW = 74,000 and 25,000,

respectively) were observed to be tightly associated with the cells. All of the intact plasminogen was removed by sublethal cell-surface trypsinization while the B' and C' proteins were not. Therefore, all cell-bound plasminogen was attached to the exterior of the cells and the B' and C' degradation products were most likely inside the cells.

2) Labeled plasminogen was degraded to molecules as small as individual amino acids, and these degradation products were found both in the incubation media and bound to the cells. The only known mechanism by which cells can mediate this kind of complete protein degradation involves lysosomal hydrolysis. Therefore, ^{125}I-plasminogen must have been internalized either by bulk phase pinocytosis (48) or endocytosis of surface-bound plasminogen. Endocytic vesicles containing plasminogen then fused with lysosomes and the plasminogen was degraded via B' and C' intermediates to low molecular weight material which included MIT. The MIT produced cannot be used for protein synthesis and was exocytosed (49).

3) The molecular weight distributions of radiolabeled molecules in the incubation media (Figure 13) were substantially different from those of the cell-associated molecules. Therefore, bulk phase pinocytosis alone cannot account for the presence of B' and C' pieces in the cells. There must be internal processing of plasminogen, most likely by lysosomal hydrolysis. It would seem that the B' and C' are formed inside the cells.

The overall pathway deduced (Figure 14) is consistent with results for both cell types. Other possible pathways seem much less probable.

It is possible that the differential effects between Balb/c SV3T3 and Balb/c 3T3 cells are due to alterations in the rates or mechanisms within this pathway. Therefore, in the ensuing discussion we retain this as the most probable situation, and consider the data in the light of other possibilities.

Plasminogen Binding

The amount of radiolabeled protein associated with 3T3 cells on a per-cell basis decreased exponentially with the time of incubation, while the amount associated with the SV3T3 cells was about the same on the third day as after 24 hours of incubation (Figure 3). Thus the overall binding to 3T3 cells decreased as the cells grew logarithmically while the amount of radiolabeled material bound to the SV3T3 cells remained relatively constant. It is therefore puzzling that no plasminogen was bound to the cell surfaces of SV3T3 after a three-day incubation. During this time, the cell population had increased about 21-fold, and the medium still contained a high concentration of plasminogen. Thus one might have reasonably expected to find that the newly-formed cells bound plasminogen at their cell surfaces in much the same way as the cells did after the first 24 hours of incubation. The 3T3 cells bound plasminogen throughout the incubation although in decreasing amounts.

There are several explanations for the decrease in cell-bound plasminogen which include:

1) SV3T3 cell-surface-bound plasminogen might have been digested by plasmin or some other serum protease thus effectively "trypsinizing" the SV3T3 cell surface at an increasing rate. This does not seem likely because trypsin (or plasmin) inhibitors such as STI did not change the amounts of plasminogen bound to either cell type. Ossowski et al. (13) found that STI did inhibit the binding of plasminogen to SVHEF, and that these cells bound substantially more than their untransformed counterparts. It is not clear whether the discrepancy between their results and the results reported here is due to differences in the cell types or in the experimental procedures. In the experiments reported here, plate washing was used throughout so as to avoid including plasminogen which was bound to the dish in the measurement of cell-associated plasminogen. It is unlikely that plasmin could have been responsible for the digestion of cell-surface-bound plasminogen. Indeed, if a serum protease had hydrolyzed plasminogen on the cell surface, it should have had a similar effect on the plasminogen in the supernatant; however, this was not the case (compare Figures 5 and 13). It seems most unlikely that plasmin, or any other serum protease, is responsible for the diminishing amounts of plasminogen on the surfaces of SV3T3 cells.

2) The surfaces and the binding characteristics of the cells may have been altered by the treatment prior to incubation in dog serum. At t = -24 hours the cells were trypsinized and plated in FCS-supplemented media. They were washed with Tris buffer, and there was a change in the type of serum supplementing the media at t = 0.

Although one can hope that the effects of these manipulations would be insignificant after the cells have passed through a complete life cycle (doubling time approximately sixteen hours), the influence of the manipulations before the incubation cannot be assessed easily and are currently unknown. These manipulations may have generated plasminogen binding sites which would have been diluted as the cells double if no new sites were generated after t = 0. The exponential decrease in the overall binding to the 3T3 cells ($t_{1/2}$ = 18 hours; Figure 3) which paralleled the cell growth ($t_{doubling}$ = 16.5 hours) is consistent with this explanation; however, the relatively constant level of overall binding to the SV3T3 cells is not.

3) There remains a somewhat remote possibility that there might be specific cell-surface receptors for plasminogen which are modulated during the incubations.

4) Plasminogen binding and processing can only be observed after unbound plasminogen has been removed. Washing the cells in suspension took several hours, therefore, interpretations of our results must be considered in the light of the opportunity the cells have had to transport, modify and perhaps eject bound plasminogen between the time they were removed from the incubations to the time the assays were conducted.

In summary, it seems inescapable that plasminogen is internalized prior to degradation. Judging from the overall rates of processing down to MIT by the cells, which are only about two times faster for SV3T3 cells than for 3T3 cells, we conclude that the initial rates of

plasminogen incorporation and cleavage to B' and C' chains must be much faster in the transformed cells.

Cellular Degradation of Plasminogen

^{125}I-plasminogen was internalized, hydrolyzed, and released into the medium by SV3T3 and 3T3 cells. The rate at which this processing occurred was approximately two times faster for the SV3T3 cells than for 3T3 cells. This is perhaps not surprising since it is well established that transport by transformed cells is generally faster than for their normal counterparts (50). It is not clear why this should be, since the growth rates for SV3T3 and 3T3 cells were similar. The intermediate products of hydrolysis had molecular weights which were similar to those of the subunits of active plasmin. This may be because it is necessary for the activating cleavage sites of zymogens to be readily accessible to enzymatic hydrolysis. During normal activation, it is important that enzymatically active molecules, rather than inactive autolysis fragments, be produced. In the case of trypsinogen activation, this enhanced susceptibility of the activating cleavage seems to be generated by the loose structure around the site of the activation cleavage rather than by any preformed substrate-like structure at that site (51,52). If this is a common feature of zymogen activation, it would not be surprising for the lysosomal hydrolases to degrade plasminogen to the level of individual amino acids via intermediates which were similar in size to the large and

small subunits of plasmin. There may be significant differences in the action of the 3T3 and SV3T3 lysosomes because rates of B' and C' degradation (Figure 6) are significantly different for the two cell types.

The Fate of Plasminogen in the Incubation

Reich and co-workers have shown that the fibrinolytic activity exhibited by many different transformed cells depends on the presence of plasminogen in the incubation medium (13), and that the cells secrete a plasminogen activator (3,4). However, the activation of plasminogen to active plasmin in the medium has so far not been shown directly. Reich (2) and Goldberg (10) have also shown that protease inhibitors, which are known to inhibit active plasmin, inhibits the protease-dependent morphological change.

In the experiments reported here, SV40-transformed 3T3 cells mediated the hydrolysis of plasminogen in the media while the untransformed cells did not (Figure 13). Presumably, the peptides produced by the hydrolysis are due to the activation of plasminogen to plasmin and subsequent serum modification of the active plasmin. All of the plasmin was found to be in the form of a >210,000 molecular weight complex, and not as free plasmin (MW = 86,000). This complex must be due to aggregation of plasmin with itself or with other serum macromolecules. There are several different plasmin inhibitors in serum (53), and it is therefore probable that a plasmin inhibitor complex is formed.

The primary inhibitor of plasmin in human serum has been shown to be a 50,000 molecular weight "anti-plasmin" which is distinct from α_1-anti-trypsin (45,46). The human plasmin-antiplasmin complex migrates on SDS denaturing gels as an approximately 130,000 dalton complex, which on reduction yields polypeptides of 72,000, 66,000 and 9,000 daltons (45,46). Müllertz and Clemmensen (46) suggest that the 9,000 dalton peptide might be a fragment of antiplasmin produced by plasmin-mediated hydrolysis.

Under reducing conditions, the labeled peptides produced from dog plasminogen are very similar to those found in the human plasmin-anti-plasmin complex. Certainly in the case of dog plasminogen the ^{125}I-labeled ∿10,000 and ∿12,000-dalton peptides are derived from plasminogen itself. The same size fragments were obtained when urokinase-activated plasminogen was added to dog serum-supplemented medium in the absence of cells (47). Therefore, cell factors are not required to produce the cleavage resulting in the 10,000 and 12,000-dalton peptides. Urokinase activation of dog plasminogen in the absence of serum does not yield these peptides (47). Together these factors suggest that an active protease, other than plasmin or cell-synthesized proteases, such as the plasminogen activator, are involved at least in producing this plasmin chain cleavage. Further investigations into this possibility are described elsewhere (47).

ACKNOWLEDGMENTS

We thank Dr. Ray Owen for the use of the Nuclear Chicago gamma spectrometer. Standard plasmin samples were provided by Dr. Alan Johnson of the American Red Cross. We thank Marlyn Teplitz and Maura Kiernan for expert technical assistance and for teaching us the necessary microbiological techniques, and Dr. Ray Teplitz for helpful advice and discussions.

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FOOTNOTES

* This work was performed with the support of National Institutes of Health Grant GM-19984 and Grant GM-24485. This paper is Contribution No. 5346 of the Norman W. Church Laboratory of Chemical Biology.

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[1] Abbreviations used: PDMC, protease-dependent morphological change; SVHEF, simian virus 40 transformed hamster embryo fibroblasts; STI, soybean trypsin inhibitor; PBS, phosphate buffer saline (0.016 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.137 M NaCl, 0.0027 M KCl, 0.00049 M $MgCl_2$, 0.0009 M $CaCl_2$, pH 7.4); 3% SDS-BME-Tris buffer, 3% sodium dodecyl sulphate Tris buffer (3% SDS, 0.1 M Tris, 2.5% glycerol, 5% 2-mercapto-ethanol, 0.002% bromophenol blue, pH 6.8); FCS, fetal calf serum; Tris buffer, isotonic Tris buffer (0.025 M Tris, 0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, 0.0005 M $MgCl_2$, 0.0009 M $CaCl_2$, pH 7.4);

DS, dog serum; PTI, pancreatic trypsin inhibitor; LMW, low molecular weight material; MIT, monoiodotyrosine, 3-iodo-L-tyrosine; TCA, trichloroacetic acid; TD buffer, isotonic Tris buffer depleted of Mg^{++} and Ca^{++} (0.025 M Tris, 0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, pH 7.4); HMW, high molecular weight material.

[2] M. J. Ross, personal communication.

TABLE I

Binding of ^{125}I-labeled Proteins to Balb/c 3T3 and Balb/c SV3T3 Cells

Molecule	Molecular Weight	Isoelectric Point	Concentration In Incubation Medium (μM)	Typical Binding (Molecules/cell)	
				Balb/c 3T3	Balb/c SV3T3
Plasminogen	94,000	6.3-8.1[a]	0.1	1×10^5	6×10^4
PTI	6,500[b]	10.5[c]	0.6	5×10^6	2×10^6
STI	22,461[d]	4.6[e]	14.6	5×10^6	1×10^6
Avidin-biotin	68,000[f]	> 10.0[g]	0.16	1×10^5	9×10^4
Ovalbumin	45,00[h]	4.5[i]	0.21	2×10^4	8×10^3

[a] Values for human plasminogen (28).
[b] See reference 33.
[c] See reference 34.
[d] See reference 35.
[e] See reference 36.
[f] See reference 37.
[g] See reference 38.
[h] See reference 39.
[i] See reference 40.

TABLE II

Effect of PDMC Inhibitors on Plasminogen Binding

Cell Type	Assay Technique	Inhibitor[a]	Plasminogrn Binding Ratios[b] (with inhibitor/without inhibitor)
Balb/c 3T3	Off-Plate	PTI	1.65 ±0.6
		PTI^{pulse}	1.15 ±0.4
		STI	1.8 ±0.2
		FCS	1.1 ±0.6
Balb/c SV3T3	Off-Plate	PTI	1.4 ±0.3
		PTI^{pulse}	0.9 ±0.2
		STI	1.15 ±0.4
		FCS	1.9 ±0.7
	On-Plate	PTI	0.4 ±0.5
SVHEF	Off-Plate	PTI	1.1 ±0.1
		PTI^{pulse}	1.2 ±0.15
		FCS	2.6 ±1.8
	On-Plate	PTI	0.45 ±0.2
		PTI^{pulse}	1.0 ±0.3
		FCS	1.5 ±1.0

[a] The inhibitors were added at t = 0 unless indicated by the superscript "pulse", in which case the inhibitor was added two hours before the binding assay.

[b] Ratios from the 24, 48, and 72-hour observations were averaged together.

TABLE III

Release of Cell-Associated Molecules

After 24, 48, and 72-hour incubations in ^{125}I-plasminogen and 10% dog serum supplemented medium which were followed by Off-Plate washings, the cells were trypsinized at 22°C or incubated in the trypsin buffer without trypsin at 22°C or 0°C for twenty minutes. The radioactivity released into the buffer was measured and expressed as a percent of the total radioactivity.

Cell Type	Incubation Time (Hrs)	Percent Radioactivity Released by:		
		Trypsin	22°C Control	0°C Control
Balb/c SV3T3	24	49.2	21.4	17.3
	48	42.2	29.1	29.4
	72	55.9	51.5	39.0
Balb/c 3T3	24	77.0	18.7	16.8
	48	63.5	28.7	25.6
	72	86.0	32.2	31.6

TABLE IV

Analysis of Low Molecular Weight Material in the Growth Media

The LMW components of the medium were separated from the macromolecules by micro-step-exclusion chromatography using Bio-Gel P-2 and then subjected to descending paper chromatography in which standard samples of MIT and I^- were co-chromatographed. All concentrations are expressed as the percent of the total amount of radioactivity in the medium.

Cell Type	Incubation Time (Hrs)	% LMW	% MIT	% I^-	% Other
Balb/c SV3T3	24	4.6	0.6	1.2	2.8
	48	5.2	1.0	1.7	2.5
	72	8.5	2.5	2.5	3.5
Balb/c 3T3	24	4.5	0.5	1.7	2.3
	48	4.3	0.5	1.8	2.0
	72	5.2	0.8	1.9	2.5

FIGURE CAPTIONS

FIGURE 1. Urokinase activation of plasminogen. 100 µl of ^{125}I-plasminogen (0.5 mg/ml in PBS) were incubated with 70 µl of urokinase (9320 plough units/ml in Tris buffer) at room temperature and after 0 (▬), 1 (——), and 60 (– – –) minutes of incubation, 10 µl aliquots were removed and mixed with 25 µl of 3% SDS-BME-Tris buffer. 10 µl of each mixture were prepared for electrophoresis and electrophoresed on 10% polyacrylamide gels as described in the text.

FIGURE 2. The effect of PTI on protease-dependent morpholigical change. Balb/c SV3T3 cells incubated in 10% dog serum-supplemented medium for 72 hours (see text for details) exhibit PDMC (a). In the presence of 6.7 µM PTI, PDMC is inhibited as can be seen by the higher density of flat substrate attached cells (b)

FIGURE 3. Time course of ^{125}I-plasminogen binding to Balb/c 3T3 and Balb/c SV3T3 cells. The cells were incubated in medium supplemented with 10% dog serum and 100 nM ^{125}I-labeled dog plasminogen and assayed for cell-associated radioactivity using the Off-Plate washing procedure described in the text. The specific radioactivity of the cells was converted to plasminogen molecule equivalents per cell based on the specific activity of the plasminogen. Each point represents the data for one plate and two to eight plates were used in each of nine separate experiments. The daily averages of all data (16-20 plates per day) are connected by the solid lines. For both types of cells, the standard deviation for replicate plates within an experiment

was about 20% of the total binding.

FIGURE 4. Time course of ^{125}I-plasminogen binding to SVHEF cells. The cells were incubated in medium supplemented with 2% dog serum and 100 nM ^{125}I-labeled dog plasminogen and assayed for cell-associated radioactivity using the Off-Plate washing procedures as described in the text. The specific radioactivity of the cells was converted to plasminogen molecule equivalents per cell based on the specific activity of the plasminogen. The width of the error bars represents two standard deviations from the mean calculated by averaging this binding curve and two binding curves in which PDMC-inhibiting concentrations of PTI (15 μM) were added at t = 0, or two hours before the binding assay. All three curves were observed during the same experiment and show that PTI had no statistically significant effect on plasminogen binding.

FIGURE 5. Electrophoretograms of SDS solubilized, β-mercaptoethanol reduced Balb/c 3T3 and Balb/c SV3T3 cells. After 24, 48, and 72 hours of incubation in ^{125}I-plasminogen and 10% dog serum-supplemented medium, the cells were washed using the Off-Plate technique, dissolved in a 3% SDS-BME-Tris buffer, boiled for three minutes, and 100-200 μl aliquots containing 5000 to 10,000 counts per minute were electrophoresed on 10% polyacrylamide gels as described in the text.

FIGURE 6. Time dependence of the relative concentrations of the Balb/c 3T3 and Balb/c SV3T3 cell-associated ^{125}I-labeled macromolecules. The peaks in electrophoretograms of solubilized and reduced Balb/c 3T3 (– – –, four experiments) and Balb/c SV3T3 (——, five experiments) cells were integrated and the relative amounts of each

macromolecular species (% of total macromolecules) were calculated. The average values and the estimated errors (width of error bars = two standard deviations) are presented as a function of incubation time.

FIGURE 7. Time dependence of the relative concentrations of the Balb/c 3T3 and Balb/c SV3T3 cell-associated ^{125}I-labeled low molecular weight material. The low molecular weight (<7000 daltons) in the electrophoretograms of solubilized and reduced Balb/c 3T3 (four experiments) and Balb/c SV3T3 (five experiments) cells was integrated and the amount of radioactivity relative to the total radioactivity in the gel was calculated. The average values and the estimated errors (width of error bars = two standard deviations) are presented as a function of incubation time.

FIGURE 8. Effect of STI on the relative concentrations of Balb/c 3T3 and Balb/c SV3T3 cell-associated ^{125}I-labeled macromolecules. After 24, 48, and 72 hours of incubation in ^{125}I-plasminogen and 10% dog serum-supplemented medium, Balb/c 3T3 (▬ ▬ ▬), Balb/c 3T3 incubated with 14.6 μM STI (– – –), Balb/c SV3T3 (▬▬▬), and Balb/c SV3T3 incubated with 14.6 μM STI (——) were prepared for electrophoresis and electrophoresed on 10% polyacrylamide gels as described in the text. The peaks in the electrophoretograms were integrated and the relative amounts of each species (% of total macromolecules) are presented as a function of time.

FIGURE 9. Cell-surface trypsinization assay. Balb/c SV3T3 cells were incubated in ^{125}I-plasminogen and 10% dog serum-supplemented medium for three days and then washed using the Off-Plate technique.

The release of radioactivity by a one-to-one solution of 10% FCS-supplemented medium:1% trypsin (w/v in PBS) was followed as a function of time (see text). An exponential function was fit to the data by the method of least squares (solid line) and showed a half-time for the trypsinization of six minutes.

FIGURE 10. Effect of trypsinization on ^{125}I-radiolabeled cell-associated molecules. After incubation in 10% dog serum, ^{125}I-plasminogen containing medium for 48 hours (Balb/c 3T3) or 24 hours (Balb/c SV3T3), the cells were harvested and washed (see text). Aliquots of the cells (Balb/c 3T3, (a); Balb/c SV3T3, (b)) were prepared for electrophoresis and electrophoresed (see text). The remaining cells were divided into three parts and incubated at 22°C in either a one-to-one solution of 10% FCS-supplemented medium: 1% trypsin (w/v in PBS) (Balb/c 3T3, (c); Balb/c SV3T3, (d)) incubated at 22°C in a one-to-one solution of 10% FCS-supplemented medium: PBS (——, Balb/c 3T3, (e); Balb/c SV3T3, (f)), or incubated at 0°C in a one-to-one solution of 10% FCS-supplemented medium: PBS (– – –, Balb/c 3T3, (e); Balb SV3T3, (f)) for twenty minutes. The mixtures were then diluted four to twenty times with TD and the cells were pelleted, dissolved in the 3% SDS-BME-Tris buffer, prepared for electrophoresis and electrophoresed (see text).

FIGURE 11. Release of Balb/c SV3T3-associated radiolabeled molecules by trypsinization. After 24, 48, and 72 hours, incubations which were followed by Off-Plate washings, Balb/c SV3T3 cells were trypsinized for twenty minutes at 22°C, and the amount of radioactivity released into the medium was measured (——). By subtracting the amount

of radioactivity released during a control experiment in which the trypsin was omitted, the amount of trypsin specific release (▬▬) was evaluated (see Table III).

FIGURE 12. Paper chromatography of low molecular weight material from the Balb/c SV3T3 medium after 72 hours of incubation. The LMW components of the medium were separated from the macromolecules by micro-step-exclusion chromatography using Bio-Gel P-2 and then subjected to descending paper chromatography. Standard samples of MIT and I^- were co-chromatographed (see text). The paper was cut and the pieces were counted to determine the mobilities (R_f) of the iodinated species relative to that of the solvent front ($R_f = 1.0$).

FIGURE 13. Electrophoretograms of Balb/c 3T3 and Balb/c SV3T3 incubation media. After 24 (——), 48 (– – –), and 72 (▬▬) hours of incubation, samples of the Balb/c 3T3 and Balb/c SV3T3 incubation media were mixed (1:1) with the 3% SDS-BME-Tris buffer, reduced and denatured. 10 µl samples were electrophoresed on 10% polyacrylamide gels as described in the text. Only one of the three electrophoretograms of the Balb/c 3T3 cell incubation media is presented because they were virtually identical.

FIGURE 14. Binding pathway. Proposed pathway of plasminogen processing by Balb/c 3T3 and Balb/c SV3T3 cells. Pg designates plasminogen.

Figure 1

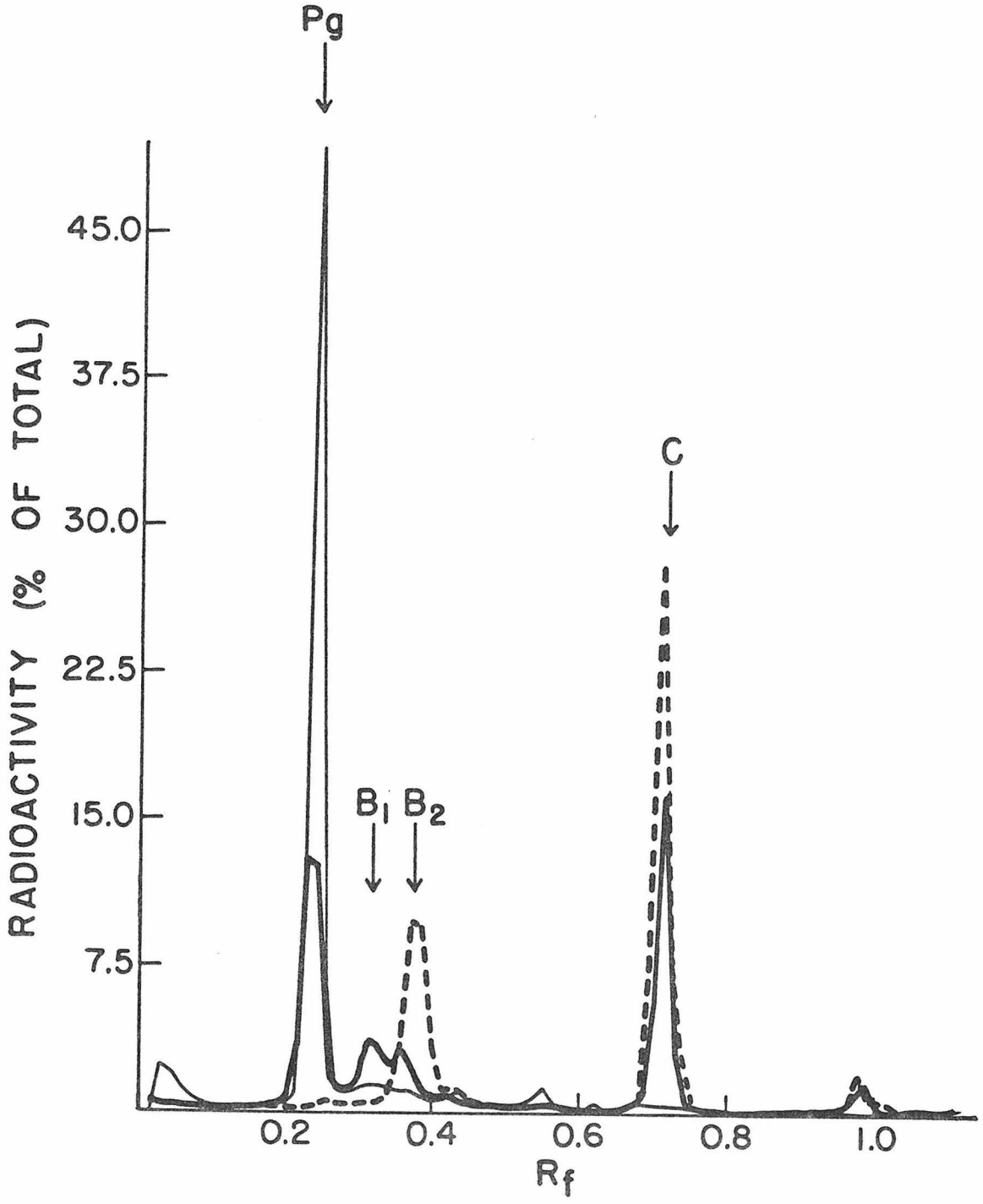

Pg
C
B1
B2
45.0
37.5
30.0
22.5
15.0
7.5
RADIOACTIVITY (% OF TOTAL)
0.2
0.4
0.6
0.8
1.0
Rf

Figure 2

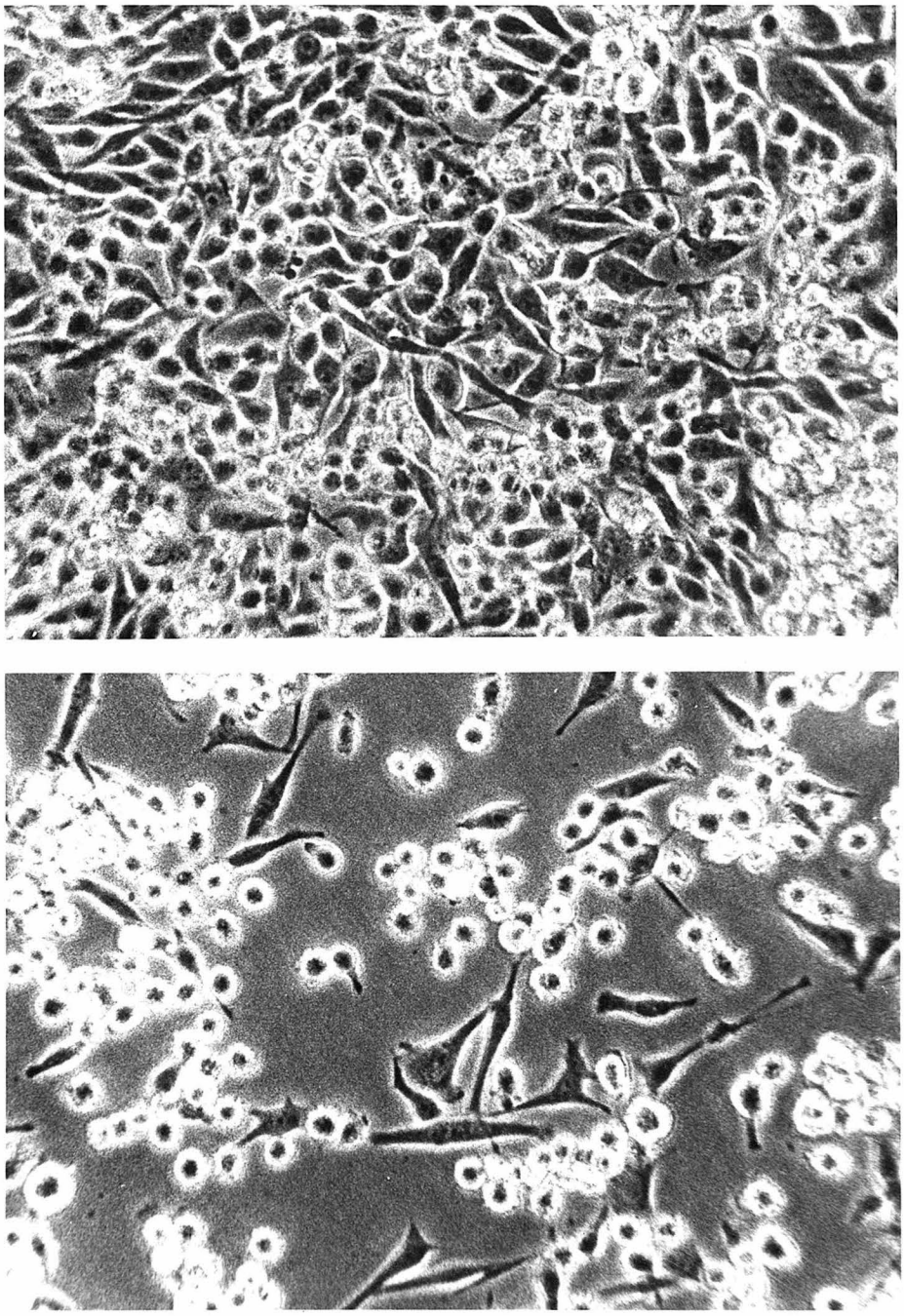


Figure 3

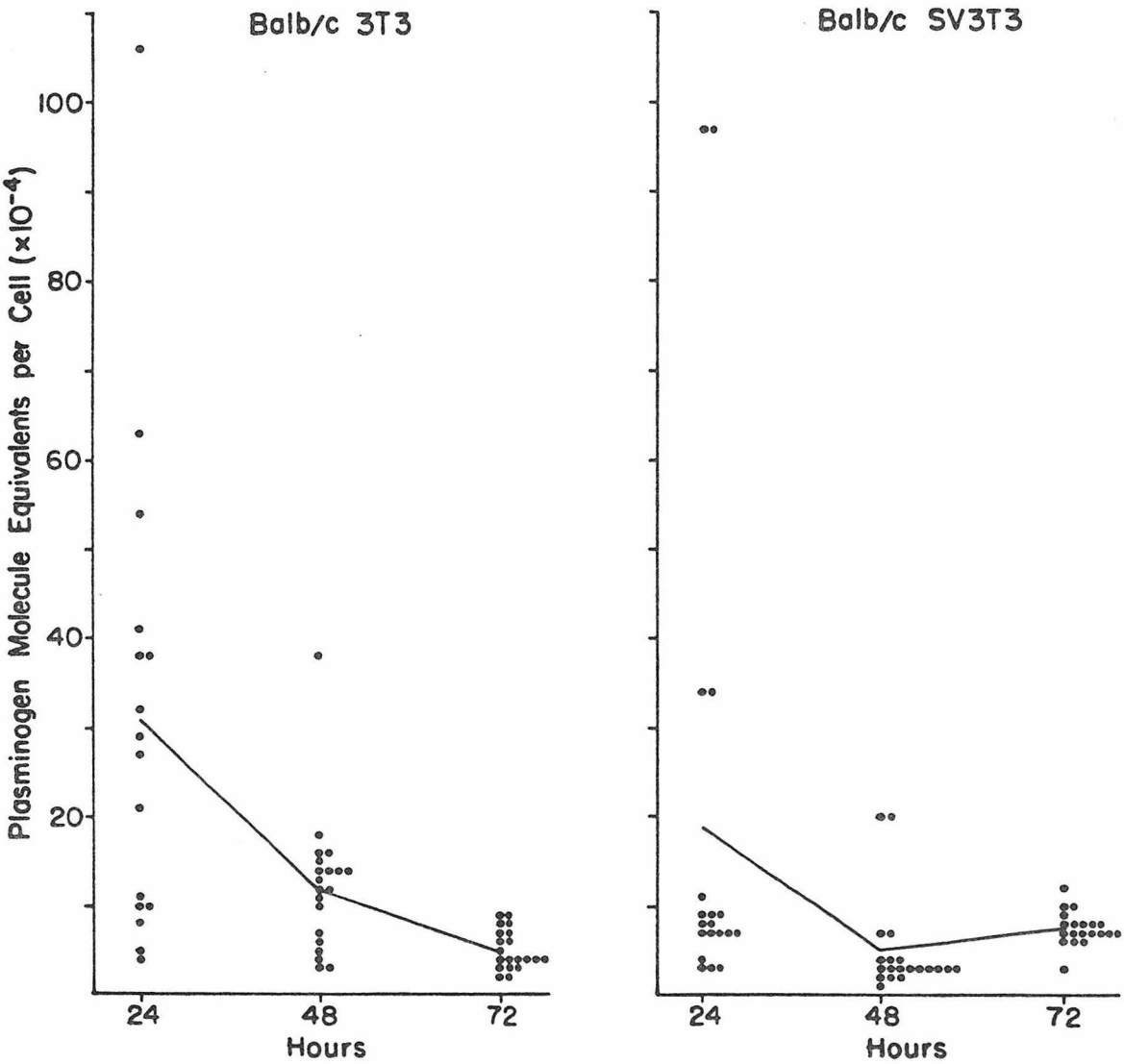

Balb/c 3T3
Balb/c SV3T3
Plasminogen Molecule Equivalents per Cell (×10⁻⁴)
100
80
60
40
20
24
48
72
Hours
24
48
72
Hours

Figure 4

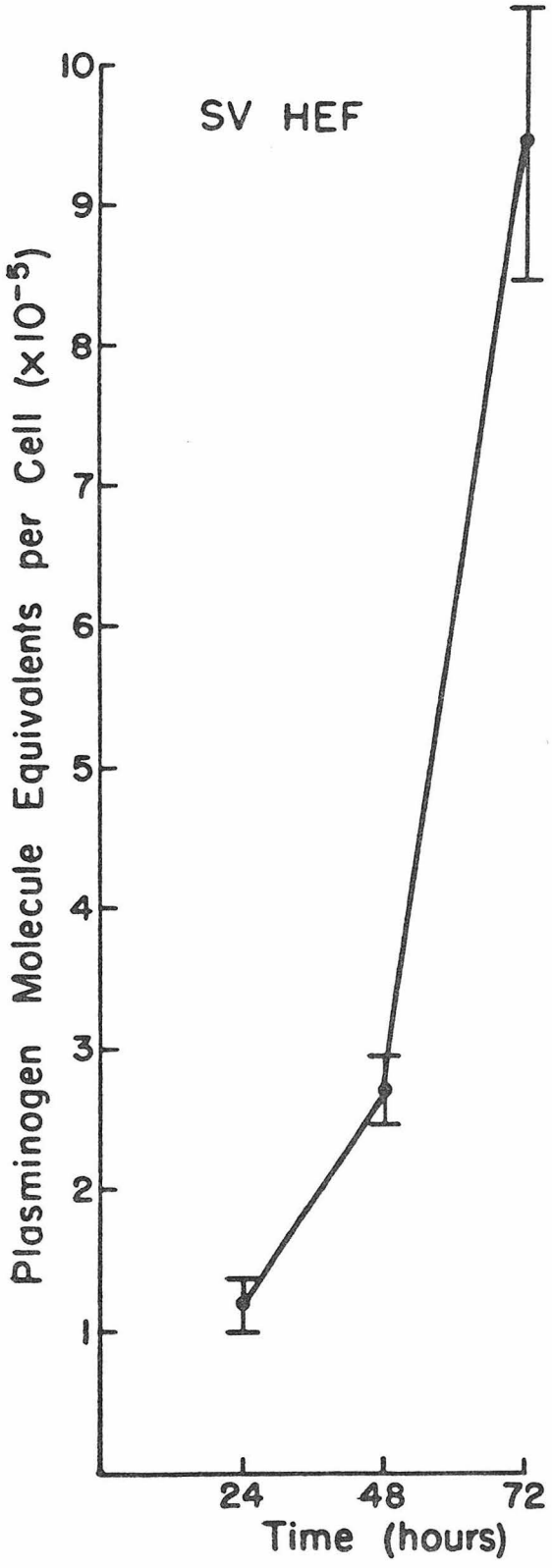

SV HEF
Plasminogen Molecule Equivalents per Cell (x10⁻⁵)
10
9
8
7
6
5
4
3
2
1
24
48
72
Time (hours)

Figure 5

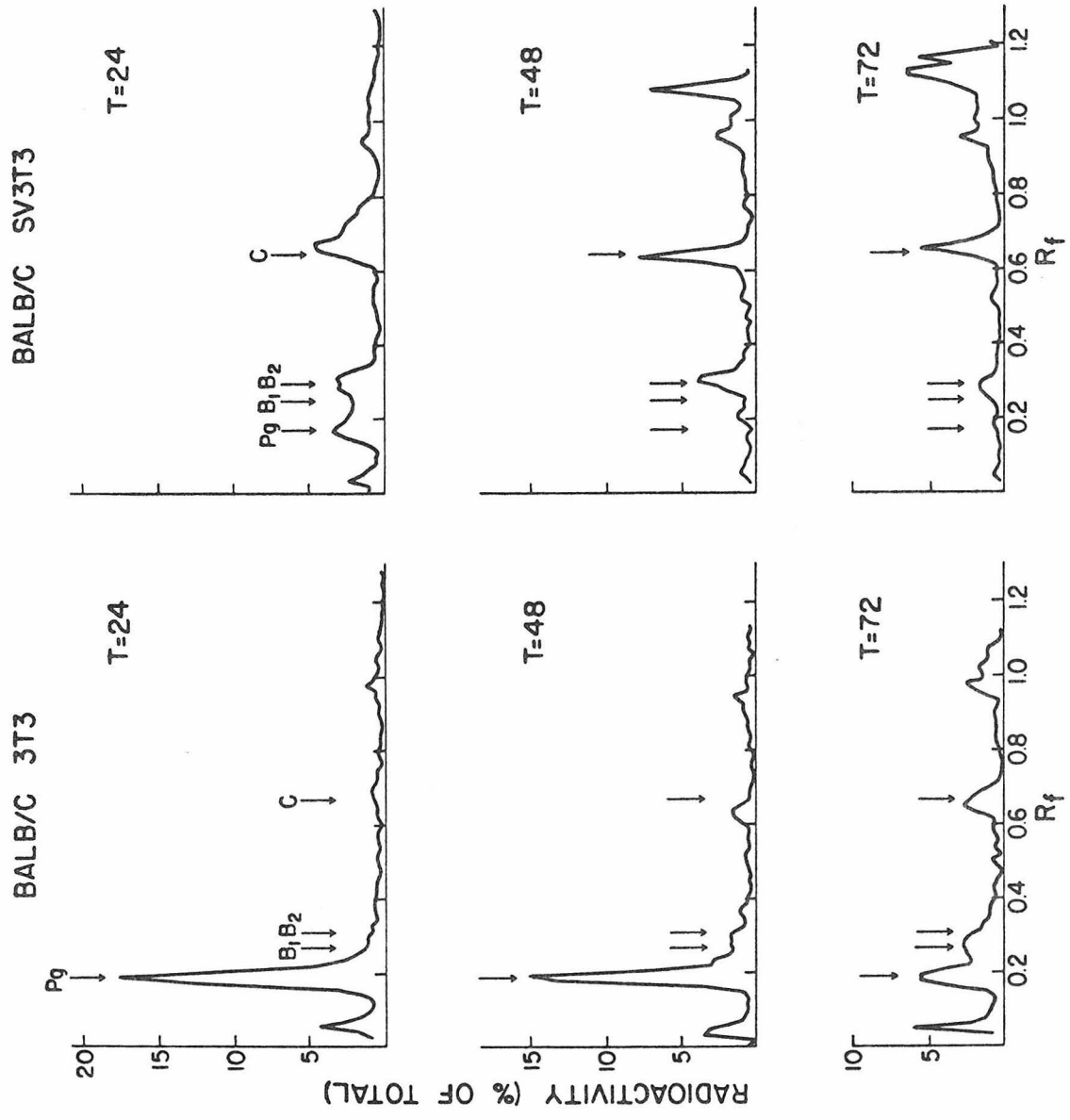

BALB/C 3T3
BALB/C SV3T3
T=24
T=48
T=72
Pg
B1B2
C
RADIOACTIVITY (% OF TOTAL)
Rf

Figure 6

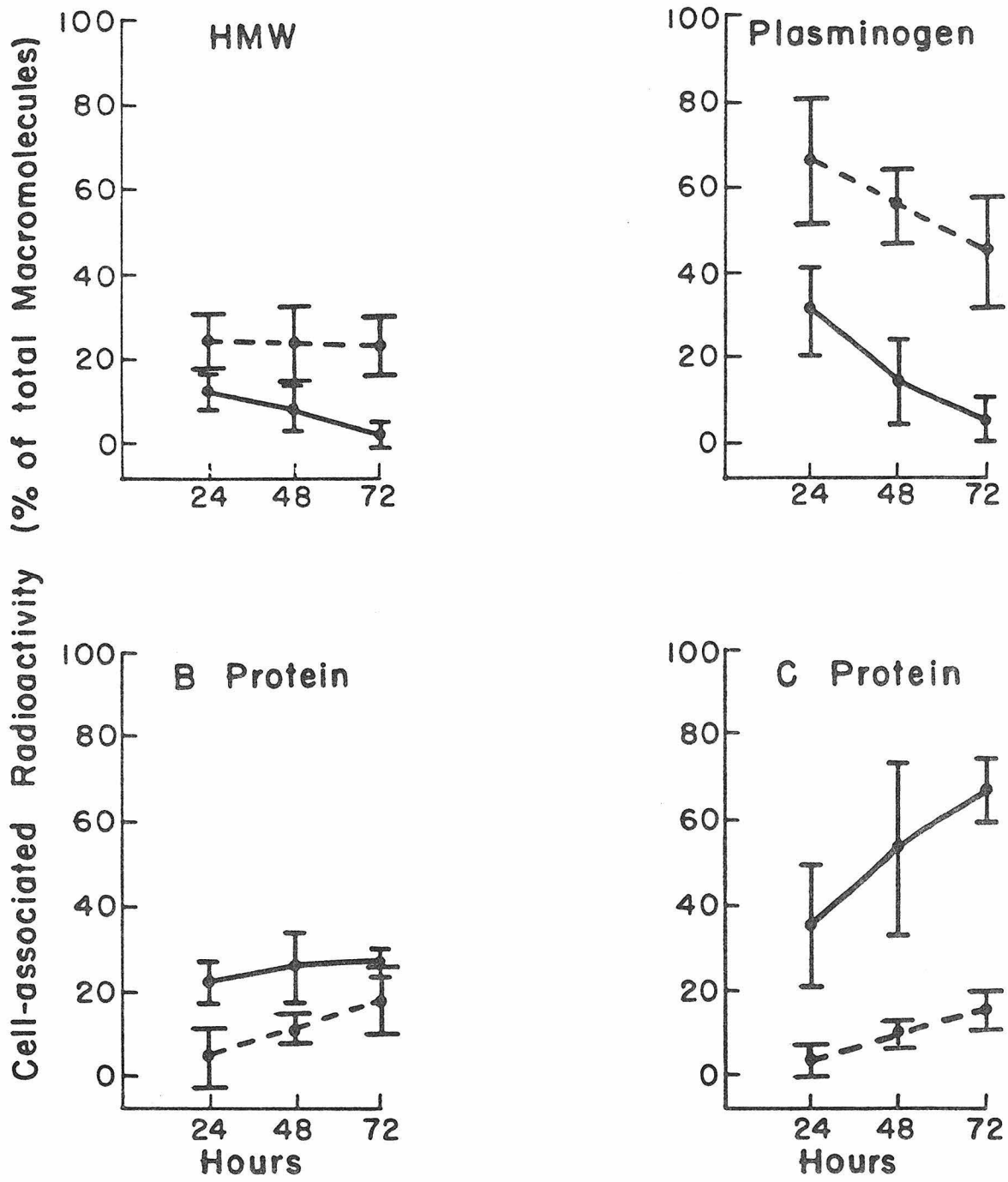

HMW
Plasminogen
B Protein
C Protein
Cell-associated Radioactivity (% of total Macromolecules)
100
80
60
40
20
0
24
48
72
Hours

Figure 7

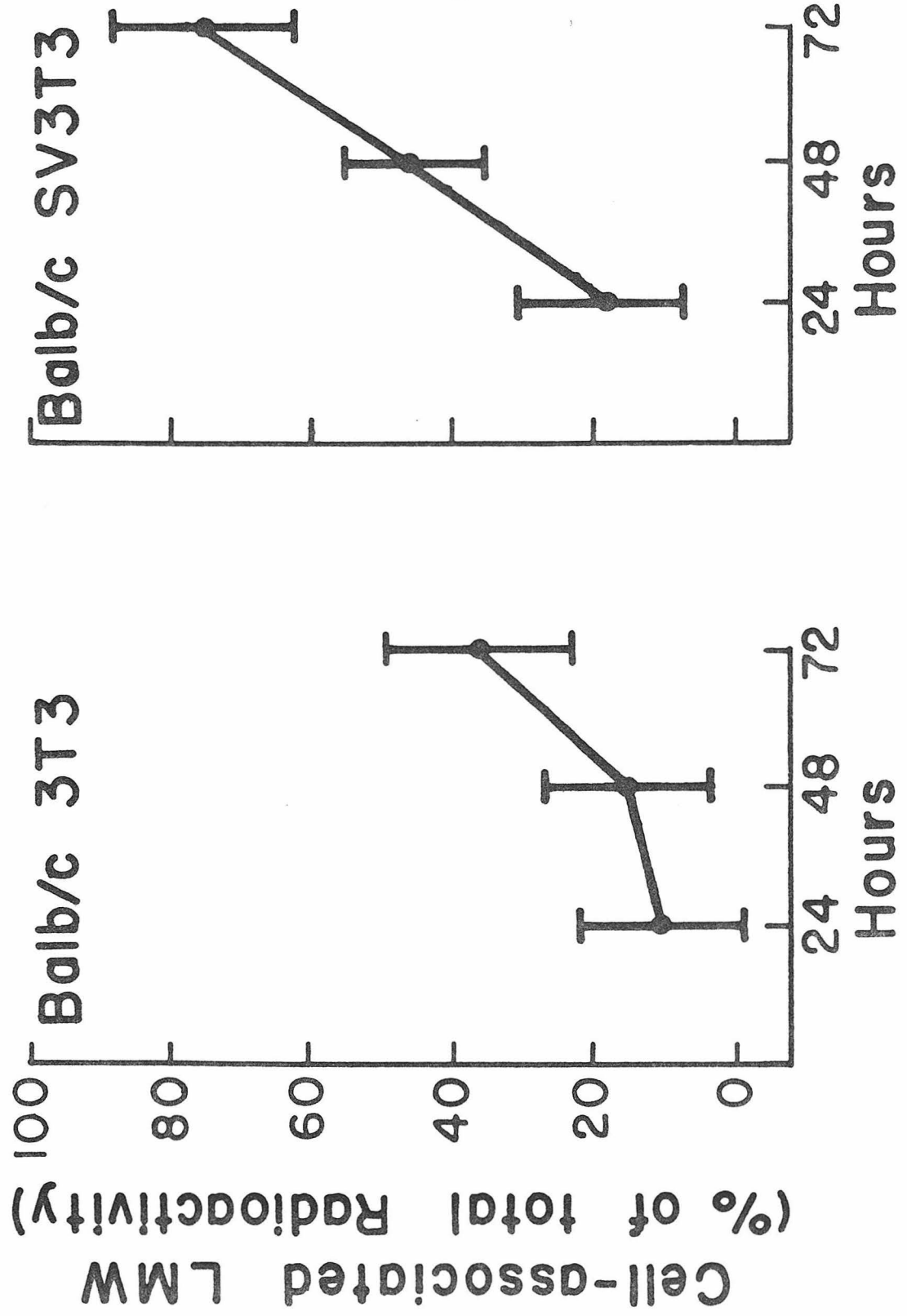

Cell-associated LMW
(% of total Radioactivity)
100
80
60
40
20
0
Balb/c 3T3
24
48
72
Hours
Balb/c SV3T3
24
48
72
Hours

Figure 8

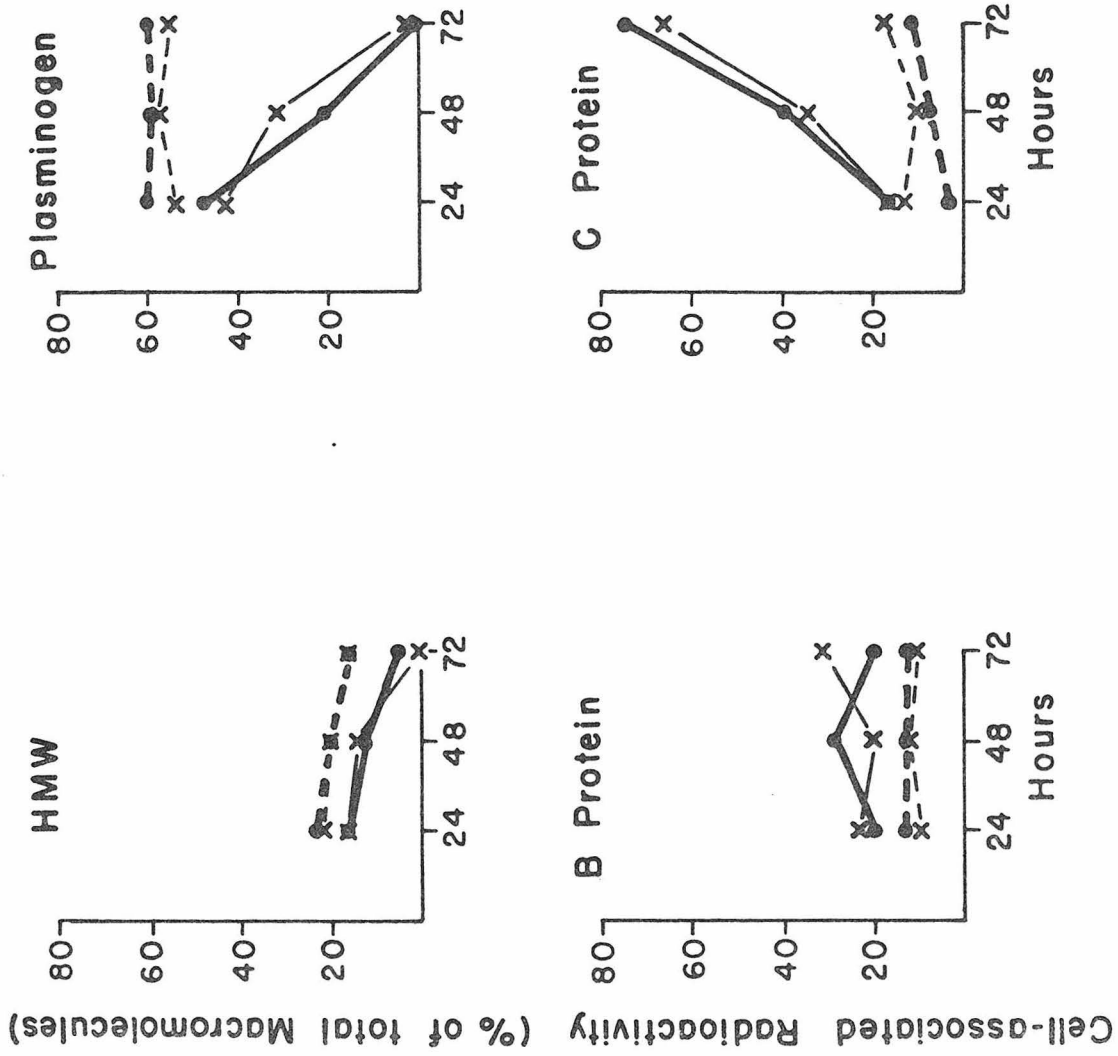

Plasminogen
C Protein
HMW
B Protein
80
60
40
20
24
48
72
Hours
(% of total Macromolecules)
Cell-associated Radioactivity

Figure 9

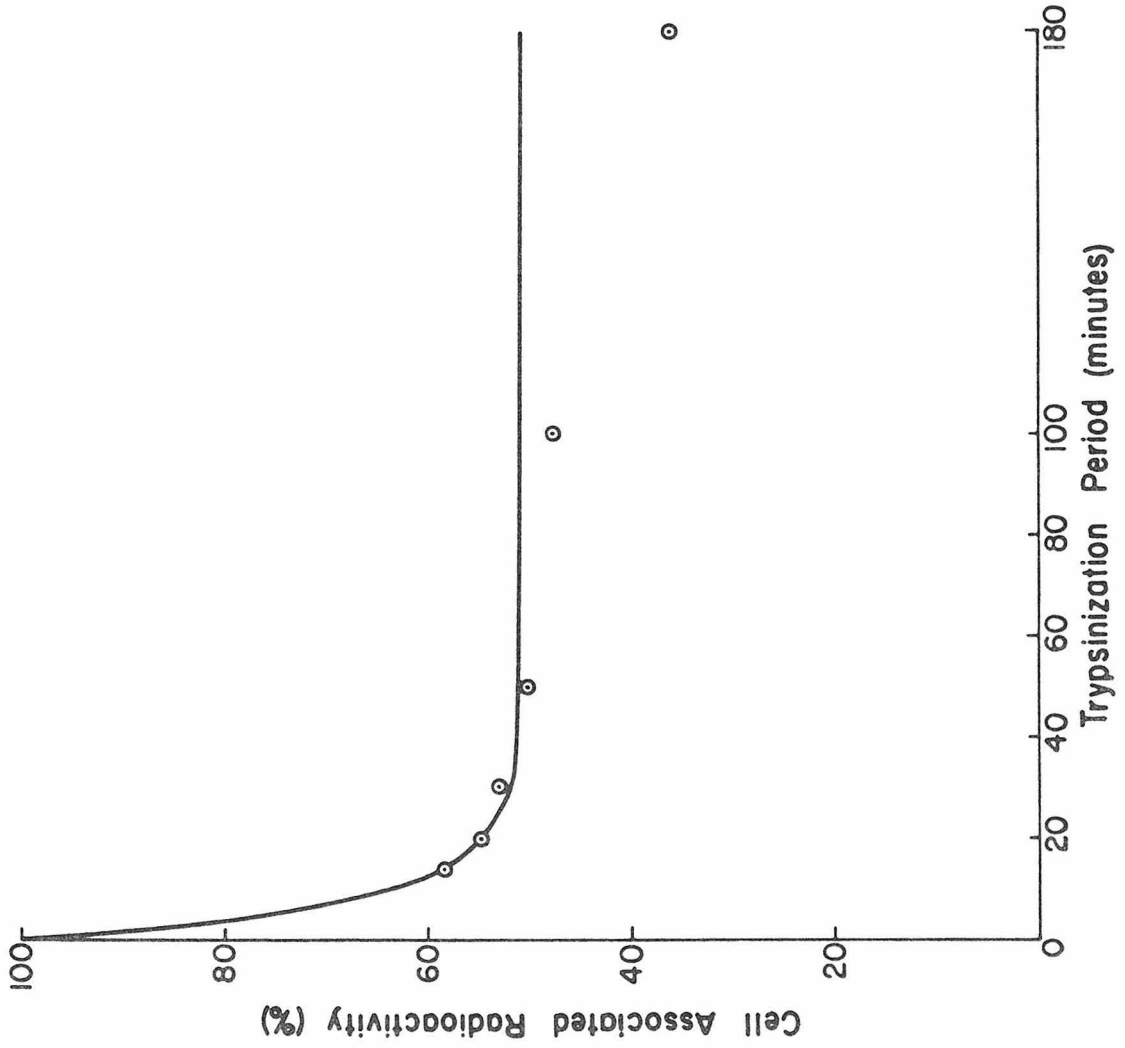

Cell Associated Radioactivity (%)
100
80
60
40
20
0
20
40
60
80
100
180
Trypsinization Period (minutes)

Figure 10

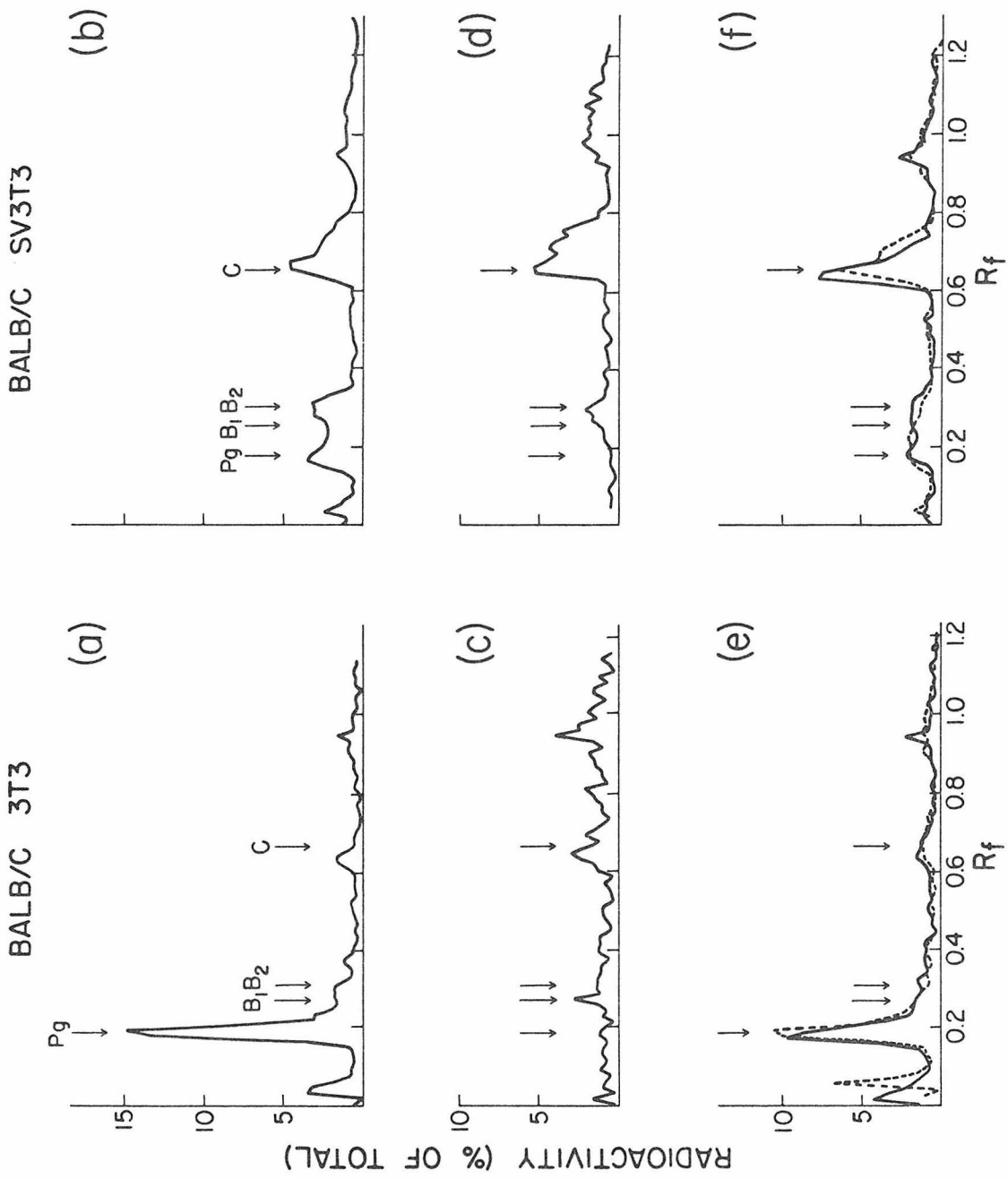

BALB/C 3T3
BALB/C SV3T3
(a)
(b)
(c)
(d)
(e)
(f)
Pg
B1B2
C
Pg B1 B2
C
RADIOACTIVITY (% OF TOTAL)
Rf
0.2
0.4
0.6
0.8
1.0
1.2
5
10
15

Figure 11

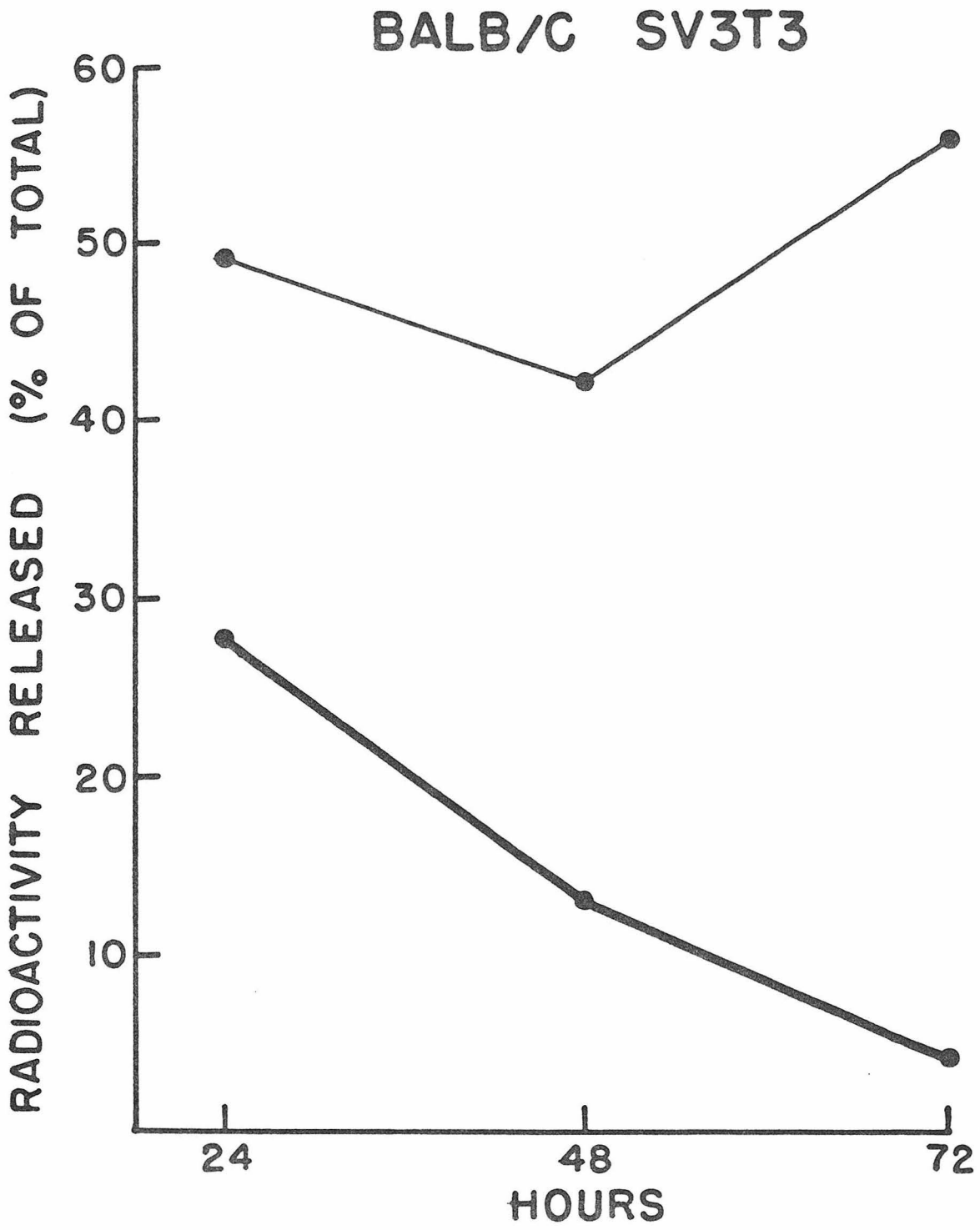

BALB/C SV3T3
RADIOACTIVITY RELEASED (% OF TOTAL)
60
50
40
30
20
10
24
48
72
HOURS

Figure 12

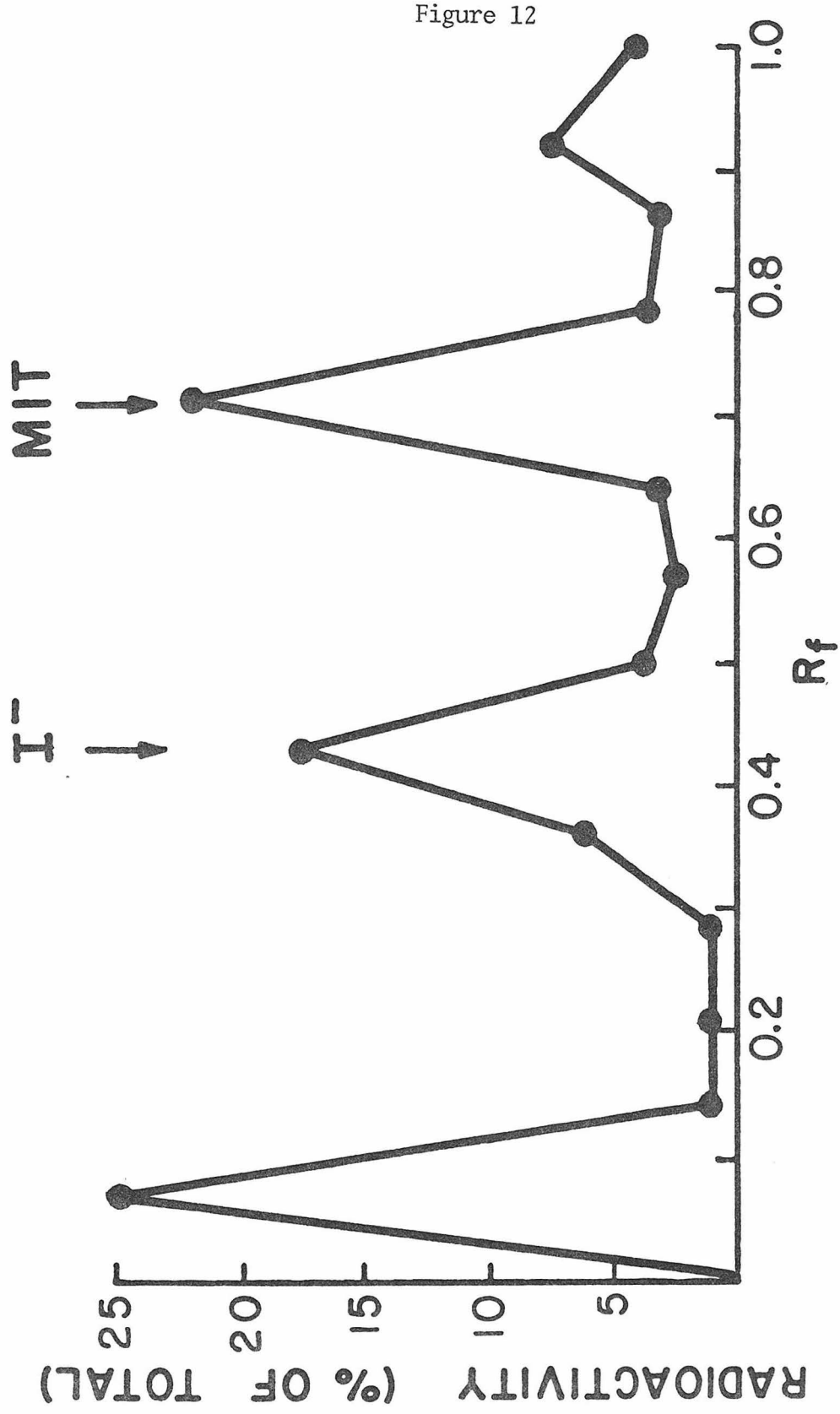


Figure 13

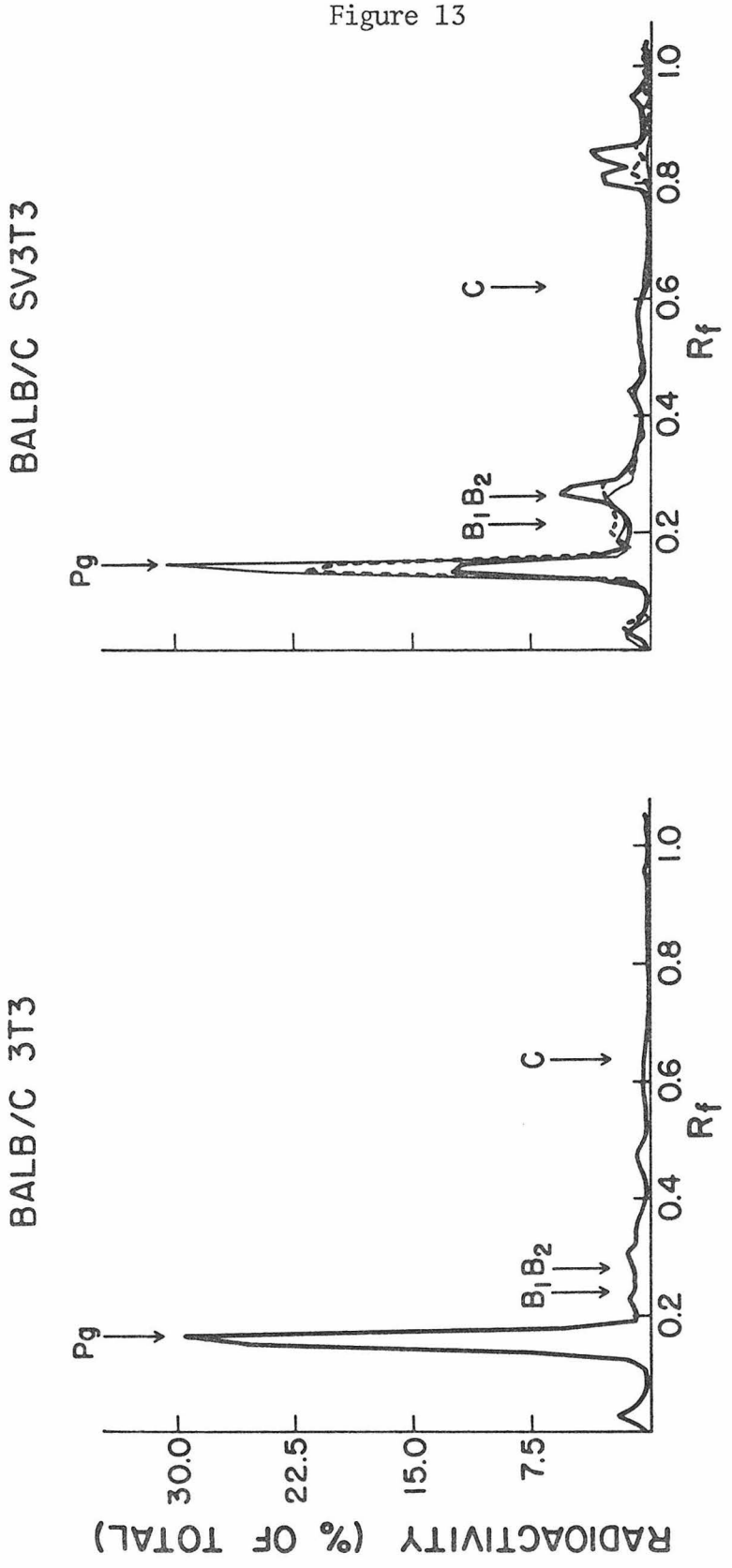
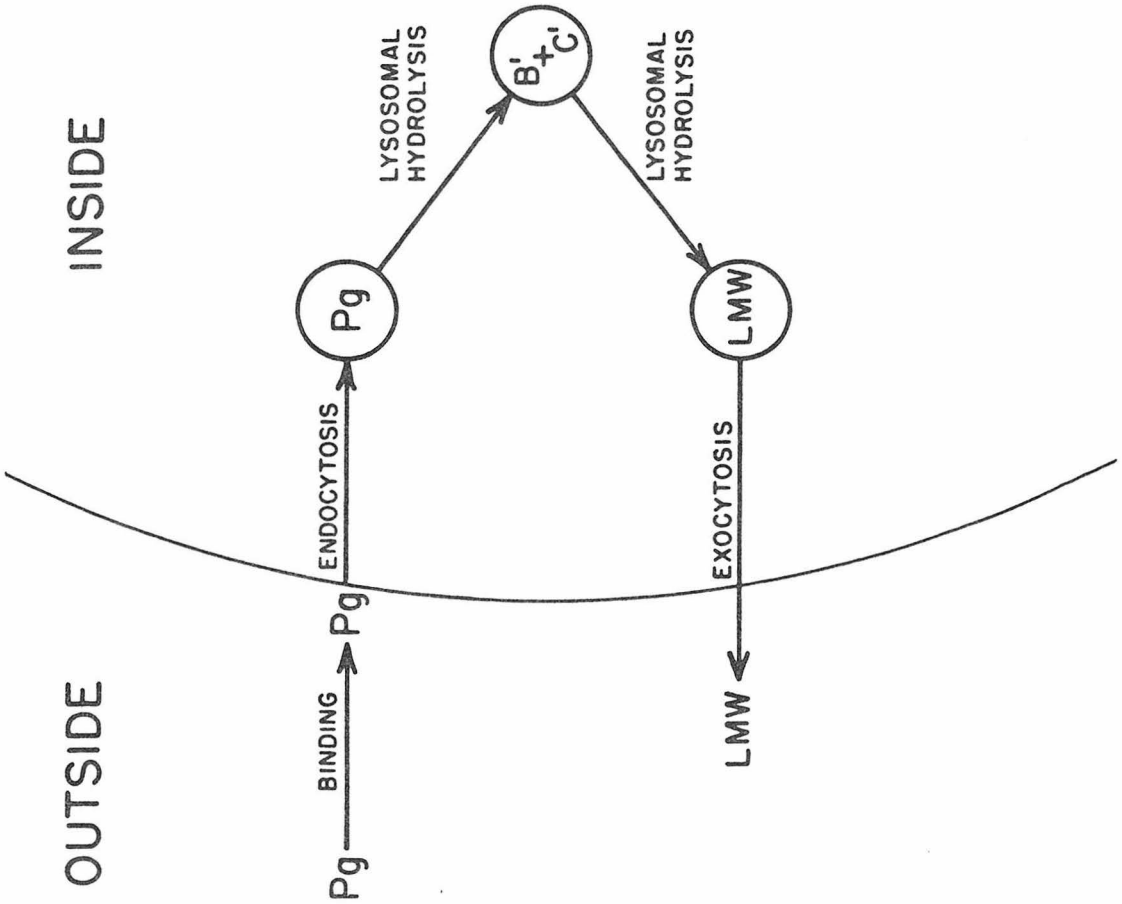

BALB/C SV3T3
BALB/C 3T3
Pg
B1 B2
C
Rf
0.2
0.4
0.6
0.8
1.0
RADIOACTIVITY (% OF TOTAL)
30.0
22.5
15.0
7.5

Figure 14


INSIDE
OUTSIDE
LYSOSOMAL HYDROLYSIS
LYSOSOMAL HYDROLYSIS
B'+C'
Pg
LMW
ENDOCYTOSIS
EXOCYTOSIS
BINDING
Pg
Pg
LMW

APPENDIX II

STUDIES ON INTERACTION OF DOG PLASMIN WITH DOG SERUM ANTIPLASMIN*

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SUMMARY

The interaction of dog plasmin with dog serum components was investigated. Through the use of a model system of urokinase *in situ* activation of plasminogen in Dulbecco's modified Eagle's medium supplemented with 10% dog serum designed to mimic the level of plasminogen activation observed in a 48-hour incubation of 10% dog serum with Balb/c SV3T3 cells in culture, it was found that some plasmin incorporated ^{32}P-labeled diisopropylfluorophosphate, implying that active plasmin exists in cell culture medium and thus may be responsible for the protease-dependent morphological change observed in these cultures. The incubation of urokinase with 10% dog serum did not activate detectable amounts of other ^{32}P-labeled diisopropylfluorophosphate inhibitable serine proteases present in dog serum. Most of the plasmin activated either by urokinase or the Balb/c SV3T3 cell derived plasminogen activator was inhibited, some by a 47,000 dalton serum component which was not dissociated from plasmin by boiling in sodium dodecyl sulfate but could be dissociated by a two-hour incubation at 37°C with hydroxylamine which suggests that it is an ester-linked covalent inhibitor similar to the antiplasmin found in human plasma.

INTRODUCTION

The enhanced proteolytic activity of cells transformed by oncogenic DNA and RNA viruses, or chemical carcinogens, which is important in mediating certain morphological changes characteristic of transformation (1,2), has been reported to be due to plasminogen activation (3,4) by a cell-derived plasminogen activator (5-12). From previous results (13) on the interaction of ^{125}I-plasminogen with Balb/c 3T3 and Balb/c SV3T3 cells in culture, it was suggested that most, and possibly all, of the plasmin formed in the Balb/c SV3T3 cell culture medium may be inhibited. We have studied the serum components responsible for this inhibition and through the use of a model system of urokinase in situ activation of plasminogen in Dulbecco's modified Eagle's medium supplemented with 10% dog serum (10% DS)[1] determined whether any active plasmin exists.

EXPERIMENTAL

Purification and Iodination of Dog Plasminogen

The procedures for purification and iodination of dog plasminogen were as described previously (13).

Cell Culture

The Balb/c SV3T3 cells used were obtained from Dr. G. Todaro and were maintained in an incubator (37°C, 5% CO_2 atmosphere) in Dulbecco's modified Eagle's medium (Gibco) with 4.5 times the glucose concentration and supplemented with 10% fetal calf serum (10% FCS). The cells were periodically checked and found free of mycoplasmal infection (14,15).

To determine the effect of incubation of SV3T3 cells on serum plasminogen, the cells were plated at 4 x 10^5 cells/60 mm petri dish (Nunclon) in 5 ml of 10% FCS-supplemented medium. After one day, the medium was removed and the cultures washed twice with isotonic Tris buffer. Fresh medium supplemented with 10% DS (Gibco) and ^{125}I-labeled dog plasminogen in phosphate buffered saline (PBS) was then added. The final concentration of ^{125}I-plasminogen was ∿100 nM.

After incubation periods of 24, 48, and 72 hours in 10% DS, the cell growth medium was removed and centrifuged at 2500 rpm for five minutes to remove any cells. The supernatants were frozen until further use.

Gel Electrophoresis and ^{125}I, ^{32}P Counting Procedure

Radiolabeled samples were electrophoresed in 5 mm diameter glass tubes on a 9 cm 10% polyacrylamide separating gel allowed to polymerize overnight which was overlaid with a 1 cm 3% stacking gel prepared

according to Laemmli (16). Reduced samples were reduced and denatured in 3% SDS-BME-Tris buffer by immersion in boiling water for three minutes immediately before electrophoresis. Nonreduced samples were denatured in 3% SDS-Tris buffer by immersion in boiling water. The volume of the sample loaded on the gels ranged from 5-200 µl. The gels were electrophoresed at 2 mA per tube for approximately five hours, frozen immediately after electrophoresis, sliced into 1 mm long pieces on a Mickle Gel Slicer (Brinkmann Instruments), and, if the samples contained ^{125}I, counted in a Beckman Biogamma II. Slices of gels containing ^{32}P were placed in glass scintillation vials with poly-seal caps (Wheaton) and heated overnight in a 45°C oven with 700 µl per vial of NCS tissue solubilizer (Amersham/Searle Lots 731 and 733) to which 56 ml of water had been added per 500 ml of NCS tissue solubilizer (9:1 solution) (17). After cooling to room temperature, 20 µl of glacial acetic acid was added to each vial followed by 10 ml of scintillation fluid (5.5 grams of Permablend III (Packard) per liter of toluene). All scintillation vials were stored in the dark before counting on a Beckman LS-250 liquid scintillation counter. Counting time per sample ranged from 5-20 minutes.

For all electrophoretograms presented, the electrophoretic mobility scale (R_F) is based on the mobility of the bromophenol blue marker dye and the radioactivity in the gel. The dependence of the R_F on molecular weight was determined using dog plasminogen (93,000 daltons) and the light chain of dog plasmin (25,000 daltons) as molecular weight standards (13). When possible these were used as

internal standards. Otherwise, samples of plasminogen and plasmin were co-electrophoresed with experimental samples as external standards.

Gel Filtration

Molecular sieve chromatography was performed on a Sephadex G-150 column (90 cm x 4.9 cm^2) equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.9. In all reported data, 7 ml (140 drop) fractions were collected with an operating pressure of 36 cm of water. The peak fractions in some cases were pooled lyophilized and the proteins re-suspended in 3% SDS-BME-Tris buffer in preparation for polyacrylamide gel electrophoresis.

A calibration curve to determine the molecular weight dependence on elution volume was obtained using aldolase from rabbit muscle (Sigma lot 105C-8690) (160,000 daltons), bovine serum albumin (Miles lot 21) (68,000 daltons), and ovalbumin from chicken egg white (Worthington lot OA35A953) (45,000 daltons).

Incubation of ^{125}I-Plasmin with 10% DS

To determine what effect incubation in 10% DS had on the electrophoretic mobility of ^{125}I-plasmin, plasminogen was activated to plasmin by incubating 100 µl of ^{125}I-plasminogen (0.5 gm/ml in PBS) with 70 µl of urokinase (Calbiochem lot 501639, 2330 Plough units/vial)

(9320 Plough units/ml in PBS) at room temperature for one hour. 30 µl of this activation mixture was then incubated with 1 ml of 10% DS (100 nM ^{125}I-plasmin) at room temperature for 30 minutes.

To ascertain whether soybean trypsin inhibitor (STI) (Worthington lot 54J358) competes with the serum component for binding to plasmin, an aliquot of the incubation mixture was incubated with 100-fold excess STI by weight for 30 minutes before the 30-minute incubation in 10% DS.

Studies on Nature of Bond Between Plasmin and the Serum Component

To determine whether the bond between plasmin and the serum component is susceptible to dissociation by hydroxylamine and therefore an ester bond, samples of 24, 48, and 72-hour SV3T3 incubation media were reduced and denatured in a 1:1 mixture with 3% SDS-BME-Tris buffer by immersion in boiling water for three minutes. These samples were either mixed 1:1 with 2 M hydroxylamine hydrochloride (Matheson, Coleman & Bell, lot 7F18) solution which was titrated to pH 7.5 with concentrated sodium hydroxide immediately before use, or mixed 1:1 with 2M sodium chloride as a control with the same ionic strength as the hydroxylamine solution. After a two-hour incubation at 37°C, these samples were mixed 1:1 with 3% SDS-BME-Tris buffer, boiled for three minutes and electrophoresed. Nonreduced samples were prepared identically except 2-mercaptoethanol was absent from all buffers used.

In situ Activation of ^{125}I-Plasminogen in 10% DS with Urokinase

To determine how much urokinase must be incubated with 10% DS to mimic the level of plasminogen activation observed in SV3T3 growth medium after two to three days of incubation, 0, 5, 10, 25, 50, and 100 µl of urokinase (9320 Plough units/ml in PBS) were incubated with 1 ml aliquots of 10% DS supplemented with 100 nM ^{125}I-plasminogen for one hour and electrophoresed.

Effect of Incubation with Urokinase on DF^{32}P Inhibitable Enzymes in 10% DS

To ascertain whether any plasmin inhibitable by ^{32}P-labeled diisopropylfluorophosphate (DF^{32}P) exists in urokinase in situ activated plasminogen in 10% DS, 2 ml of 10% DS supplemented with 100 nM dog plasminogen was incubated with 20 µl of urokinase (9320 Plough units/ml in PBS) at room temperature for one hour. 200 µl of DF^{32}P (Amersham/Searle, lot 192, 71 m Ci/m mol) solution (16.6 mg DF^{32}P/ml of 2-propanol) was then added and incubated at room temperature for one hour with constant stirring. The sample was dialyzed overnight at 4° C against two changes of a 300:1 volume ratio of isotonic Tris buffer.

So that the extent of plasminogen activation by urokinase could be determined, a sample treated identically to that described above was supplemented with 100 nM ^{125}I-plasminogen rather than 100 nM plasminogen.

To determine which components in 10% DS incorporate $DF^{32}P$ without pre-incubation with urokinase, a sample was treated identically to the first sample described above, except the initial one-hour incubation was not supplemented with exogenous urokinase and plasminogen.

To detect what levels of $DF^{32}P$ incorporation was attained in urokinase and plasmin in the absence of 10% DS, a 2 ml aliquot of a 100 nM plasminogen solution in PBS was incubated with 20 µl of the urokinase solution for one hour at room temperature. 100 µl of the $DF^{32}P$ solution was incubated at room temperature for one hour with constant stirring. The sample was dialyzed as described previously.

RESULTS

Characterization of Serum Component-Plasmin Complex

Interaction of Urokinase Activated-Plasminogen with Serum

Dog plasminogen has been shown to be a single polypeptide chain of about 93,000 daltons (13). During urokinase activation, two peptide bonds in plasminogen are cleaved (13,18). One cleavage yields a two-chained, disulfide-linked molecule with the smaller active-site-containing subunit (25,000 daltons) derived from the carboxyl terminus of plasminogen. The second cleavage which occurs near the N-terminus releases a 6,000-8,000 dalton peptide.

The polypeptides produced when dog plasminogen is activated to plasmin by urokinase are referred to as B_1, the plasmin heavy chain (MW 74,100) which is disulfide linked to C, the plasmin light chain (MW 25,000) and B_2, the plasmin heavy chain derived from B_1 after the N-terminal cleavage which accompanies urokinase activation (13).

^{125}I-plasminogen is completely activated to ^{125}I-plasmin by incubating with urokinase for one hour (Figure 1A). If ^{125}I-plasmin is incubated with Dulbecco's modified Eagle's medium supplemented with 10% DS, the electrophoretic characteristics of the radiolabeled peptides are changed (Figure 1B). A comparison of the electrophoretogram of plasmin incubated with 10% DS with the electrophoretogram of the original plasmin solution (Figure 1A,B) reveals a dramatic decrease in

the amount of C protein after the incubation. This is accompanied by the formation of a low molecular weight doublet (D protein) and a broadening of the B_2 protein peak. The decrease in the C protein peak is apparently due to two modes of modification. The C protein is degraded to smaller polypeptides of molecular weight 10,000 and 12,000 (D protein) and some of the C protein is complexed with a serum component such that its electrophoretic mobility becomes slightly less than the B_2 protein, thus producing the observed shoulder (Figure 1B).

The molecular weight of the B_2 protein shoulder due to the C protein-serum component complex is determined to be 72,000. Since the C protein has a molecular weight of 25,000, the apparent molecular weight of the serum component is 47,000, which is similar to the molecular weight of the antiplasmin found in human plasma (19-21).

If the serum component which complexes with the C protein binds in the catalytic binding pocket, plasmin inhibitors should compete with the serum component for binding with the C protein. To determine whether the serum component would bind to plasmin which is inhibited with STI, ^{125}I-plasmin was incubated with excess STI before being incubated in 10% DS. The electrophoretograms of the original plasmin (Figure 1A) and of STI-inhibited plasmin incubated with 10% DS (Figure 1C) are virtually identical. Thus STI prevents the serum component from binding to the C protein implying that the serum component and STI bind to the same site on plasmin, and that the serum component is a plasmin inhibitor.

The inhibition of ^{125}I-plasmin with STI before incubation in 10% DS not only prevents the formation of the C protein-serum component complex, but also prevents the formation of C protein degradation products (D protein) (Figure 1C).

Studies on Balb/c SV3T3 Cell Supernatants

When SV3T3 cells are grown in medium supplemented with ^{125}I-plasminogen, some of the plasminogen is activated to plasmin, presumably by the cell derived plasminogen activator (13). From the very small amount of C protein observed on electrophoretograms of the SV3T3 growth medium (Figure 2), one may conclude that the C subunit of plasmin is modified by degradation to the 10,000 and 12,000 dalton species (D protein) and by binding to the 47,000 dalton serum component similar to what is observed when urokinase activated plasminogen is incubated with 10% DS.

These data suggest that the plasmin in the SV3T3 growth medium is in three forms: 1) the heavy chain (B_2) disulfide linked to the light chain (C) [native plasmin]; 2) the heavy chain (B_2) disulfide linked to the split light chain (D) [degraded plasmin]; and 3) the heavy chain (B_2) disulfide linked to the light chain (C) which in turn is bound to the serum component [complexed plasmin]. The native plasmin and degraded plasmin forms may also be complexed with serum components, but these complexes are dissociated in SDS.

If nonreduced samples of the 24, 48, and 72-hour incubations of SV3T3 growth media supplemented with ^{125}I-plasminogen are electrophoresed, three radiolabeled species are observed (Figure 3). These have an apparent molecular weight of 153,000, 127,000 and 94,000. The 153,000 and 127,000 dalton species, which are not dissociated in SDS, may be plasmin-serum component complexes. The 94,000 species is presumably plasminogen. The amount of the 153,000 dalton species increases significantly between the second and third day of incubation, while the amount of the 127,000 dalton species increases gradually over the three days of incubation.

The electrophoretic characteristics of the plasmin-serum component complex does not offer any information as to what plasmin species exist in solution. To acquire this information, samples of the 24, 48, and 72-hour incubation media supplemented with ^{125}I-plasminogen from SV3T3 cultures were fractionated on a Sephadex G-150 column. The SV3T3 growth medium migrates as two radiolabeled peaks on a Sephadex G-150 column (Figure 4), with the apparent molecular weights of 212,200 ±5,500 and 98,000 ±5,650, where the errors represent the standard deviation determined from four independent chromatograms.

During the three days of incubation with SV3T3 cells, the amount of the 98,000 dalton species decreases while the amount of the 212,200 dalton species increases. This is similar to the observed decrease of the plasminogen peak and increase of plasmin peaks on electrophoretograms of reduced and denatured SV3T3 cell media during the three-day incubation period (Figure 2). To compare the chromatographic data

with the electrophoretic data, the radioactivity under the 98,000 dalton peak on each chromatogram was determined and expressed as a percent of the total radioactivity. Similarly, the area under the plasminogen peak on each electrophoretogram was determined and expressed as a percent of the total radioactivity on the electrophoretogram. The good agreement (Table I) of the percent radioactivity under the 98,000 dalton peak from the chromatograms with the percent of radioactivity which is plasminogen on a reduced and denatured gel, suggests that the 98,000 dalton peak is essentially all plasminogen and that the 212,200 dalton peak is essentially all plasmin.

To further characterize the radiolabeled species contained in the 212,200 dalton and 98,000 dalton peaks observed on the chromatogram of 72-hour SV3T3 growth media, the peak fractions were pooled, lyophilized, and electrophoresed. As predicted, the 98,000 dalton material is primarily plasminogen with a small amount of plasmin (Figure 5B). The electrophoretogram of the 212,200 dalton material (Figure 5A), when reduced and denatured, has six major radiolabeled peaks. Based upon their molecular weights, these peaks are identified as: plasminogen, B_2 protein and C protein-serum component complex, C protein, D protein produced when the C protein is split, and some low molecular weight material. Thus, the 212,200 dalton species is primarily plasmin in various forms and a small amount of plasminogen.

Nature of Serum Component Binding to Plasmin

The fact that the plasmin-serum component complex, whether reduced or nonreduced, is not dissociated when denatured by SDS suggests that a covalent bond may be formed between the inhibitor and the C protein of plasmin. The covalent bond may be a carboxylic ester formed between the serine in the active site of plasmin and the carboxyl terminus of the inhibitor as has been proposed for the plasmin-human antiplasmin complex (19-21). To test this possibility, both reduced and nonreduced, denatured samples of 24, 48, and 72-hour SV3T3 incubation media were incubated with hydroxylamine, a nucleophile, which should break any ester bonds between plasmin and the serum component. As a control similar incubations were performed in sodium chloride of the same ionic strength as used in the hydroxylamine incubations.

The incubation in sodium chloride had no effect on the 153,000, 127,000 and 94,000 dalton peaks of nonreduced samples of SV3T3 growth media (Figures 6A,B,C, and Figure 3). The incubation with hydroylamine caused the disappearance of the 153,000 and 127,000 dalton peaks in electrophoretograms of nonreduced samples and the concomitant increase in a plasminogen shoulder which is presumed to be plasmin (Figure 6D,E,F). Thus, incubation with hydroxylamine apparently causes the dissociation of the serum component from plasmin changing the electrophoretic mobility of the radiolabeled species from that of a 153,000 and 127,000 dalton plasmin-serum component complex to that

of a ∿90,000 dalton plasmin molecule.

As is found for nonreduced samples, incubation of reduced samples with sodium chloride had no effect on the electrophoretic mobility of the radiolabeled species of SV3T3 growth media (Figure 7A,B,C and Figure 2). In reduced samples, the serum component is complexed with the C protein of plasmin, and the complex has an electrophoretic mobility similar to that of the B_2 protein of plasmin. When reduced samples of SV3T3 growth media are incubated with hydroxylamine, the peak marked B_2 in the electrophoretograms becomes less broad and the amount of C protein increases (Figure 7D,E,F). Thus, the C protein-serum component complex which migrates near the B_2 protein of plasmin is apparently dissociated when incubated with hydroxylamine, causing an increase in the C protein material.

The data from both reduced and nonreduced samples of SV3T3 growth media are therefore consistent with the plasmin-serum component complex being a covalent, ester-linked complex which is dissociable when incubated with a nucleophile such as hydroxylamine.

Effect of Preincubation with Urokinase on $DF^{32}P$ Inhibitable Enzymes in 10% DS

Even though some of the plasminogen in SV3T3 growth medium is activated to plasmin, essentially all radiolabeled material with the mobility of the 25,000 dalton, active-site-containing subunit of plasmin is absent from electrophoretograms of reduced and denatured

samples (Figure 2). This observation begs the question whether any active plasmin exists in SV3T3 growth medium.

To facilitate the resolution of this question, a model system of urokinase in situ activation of plasminogen in 10% DS which would mimic the level of plasminogen activation observed in SV3T3 growth medium after two to three days of incubation was developed. The electrophoretograms of 10% DS supplemented with ^{125}I-plasminogen and incubated with various concentrations of urokinase (Figure 8) contain the peaks characteristic of conversion of plasminogen to the three forms of plasmin observed in electrophoretograms of SV3T3 growth medium (Figure 2).

To aid in the comparison of the level of plasminogen activation in SV3T3 cultures to that in urokinase incubation, the area under the peaks in the electrophoretograms of SV3T3 growth medium (Figure 2) and urokinase in situ activation of plasminogen (Figure 8) were determined. The amount of plasminogen which was activated to plasmin may be determined from the percent of the total counts on each electrophoretogram which is under the plasminogen peak, and from the original ^{125}I-plasminogen concentration (100 nM), the concentration of plasmin may be determined. The concentration of each of the plasmin forms also may be calculated,[2] and the results for the SV3T3 growth media and the urokinase in situ activation of plasminogen are presented in Tables II and III, respectively.

By comparing Table III with Table II, one observes that a one-hour incubation of 93.2 Plough units/ml of urokinase with 10% DS

results in a plasmin activation level and distribution of plasmin species very similar to that of a 48-hour incubation of 10% DS with SV3T3 cells. So this urokinase concentration was adopted for the studies which follow.

It is also of interest to note in Table II that the degraded plasmin concentration in the growth medium of SV3T3 cells increases dramatically between the second and third day of growth. This increase is accompanied by a decrease in the complexed plasmin concentration. A similar increase in degraded plasmin concentration with a concomitant decrease in complexed plasmin concentration is observed in the urokinase in situ activation of plasminogen in 10% DS at high total plasmin concentrations (>50 nM) (Table III).

To resolve the question whether any active plasmin exists in the model system and thus in SV3T3 growth medium, the urokinase incubation with 10% DS was followed by a one-hour incubation with ^{32}P-labeled diisopropylfluorophosphate. DF^{32}P binds covalently to the active-site serine of serine proteases, so any active serine protease should become radiolabeled with ^{32}P including urokinase and plasmin. An electrophoretogram (Figure 9, dark line) of a reduced sample of 10% DS incubated with urokinase and then with DF^{32}P contains five major substances which incorporated DF^{32}P. These include a high molecular weight material which only slightly entered the gel, a 60,000 dalton species, a 47,900 dalton species, a 25,000 dalton species, and a 23,500 dalton species.

To determine which of these peaks are due to the urokinase incubation, a reduced sample of 10% DS which was incubated with $DF^{32}P$ without preincubation with urokinase was electrophoresed (Figure 9, light line). The electrophoretogram contains the same five major peaks; however, the electrophoretogram of the sample preincubated with urokinase differs from the electrophoretogram of the sample which was not preincubated with urokinase by the existence of a 29,000 dalton peak and an increase in the 25,000 dalton peak.

The 29,000 and 25,000 dalton peaks are also observed on electrophoretograms (Figure 10A) of reduced samples of urokinase incubated with plasminogen in the absence of 10% DS followed by an incubation with $DF^{32}P$. The 25,000 dalton peak is presumably the active-site containing subunit of plasmin while the 29,000 dalton peak is the active-site containing subunit of urokinase which has been reported to have a molecular weight of 28,000 (22).

To determine if indeed the increase in the 25,000 dalton peak in reduced samples of 10% DS preincubated with urokinase is due to the C protein of plasmin, nonreduced electrophoretograms in which plasmin migrates as a 94,000 dalton species were obtained. Electrophoretograms (Figure 11, light line) of 10% DS not preincubated with urokinase contain three major substances which incorporated $DF^{32}P$. These include a high molecular weight material, a 60,000 dalton species, and a 23,000 dalton species. An electrophoretogram (Figure 11, dark line) of 10% DS which was preincubated with urokinase contains the same three substances and in addition a 32,000 dalton peak and a

94,000 dalton peak. From electrophoretograms of nonreduced samples of urokinase incubated with plasminogen in the absence of 10% DS (Figure 10B), the 94,000 dalton peak is identified as plasmin and the 32,000 dalton peak is presumably the active proteolytic fragment of urokinase reported to have a molecular weight of 31,500 (23).

Therefore, by comparing electrophoretograms of both reduced and nonreduced samples of 10% DS which were preincubated with urokinase to samples which were not exposed to urokinase, a net incorporation of $DF^{32}P$ is observed in plasmin-sized material in those samples preincubated with urokinase suggesting that active plasmin exists in solution in this model system and thus presumably in SV3T3 growth medium.

In what form does this $DF^{32}P$ inhibitable plasmin (active plasmin) exist in solution? From chromatographic and electrophoretic data of SV3T3 growth media (Figures 4 & 5), it is known that most of the plasmin-sized material in these cultures migrates with a 212,200 dalton peak of a Sephadex G-150 column while a small amount of plasmin migrates with the 98,000 dalton peak. These observations were confirmed for urokinase in situ activated ^{125}I-plasminogen.[3] To learn in which of these chromatographic peaks $DF^{32}P$ inhibitable plasmin migrates, samples of 10% DS incubated with $DF^{32}P$, both with and without preincubation with urokinase, were fractionated on a Sephadex G-150 column. Those fractions known to comprise the plasmin-plasminogen peaks from a previous fractionation of an identical sample which contained ^{125}I-plasminogen (Figure 12) were pooled and electrophoresed. It should be noted that the chromatogram of the in situ urokinase

activated ^{125}I-plasminogen (Figure 12) differs from the chromatograms of SV3T3 growth media (Figure 4) by the appearance of material in the void volume. This void volume peak in addition to the 212,200 peak and the 98,000 peak was investigated for plasmin activity.

Electrophoretograms of reduced samples of the fractions comprising the void volume peak of 10% DS incubated with DF^{32}P and either preincubated or not preincubated with urokinase had no ^{32}P containing peaks. Thus no active serine proteases, including plasmin, migrate in the void volume peak.

The electrophoretograms of a reduced sample of the 212,200 dalton peak material, obtained when a sample of 10% DS incubated with DF^{32}P was fractionated, have two ^{32}P containing peaks (Figure 13A) with relative molecular weights of 47,900 and 25,000. The corresponding electrophoretogram of the fractionated material of a sample which had been preincubated with urokinase has the same two peaks in the same relative proportion (Figure 13A) indicating that urokinase incubation had no effect on the serum components which incorporate DF^{32}P and fractionate as a 212,200 dalton peak. To ascertain whether the 25,000 dalton peak in these electrophoretograms is due to plasmin, nonreduced samples in which plasmin would exist as a ∿94,000 dalton molecule were electrophoresed. No peak is observed in the region of the electrophoretograms (Figure 13B) where plasmin would migrate (R_F = 0.28). So within the detectability of this experiment none of the plasmin in the 212,200 dalton peak incorporates DF^{32}P and therefore no active plasmin exists in this peak.

The electrophoretogram of a reduced sample of the 98,000 dalton peak material obtained when a sample of 10% DS incubated with DF^{32}P was fractionated has one ^{32}P containing peak (Figure 14A) with a molecular weight of 60,000. The corresponding electrophoretogram (Figure 14B) of the fractionated material of a sample which had been preincubated with urokinase has the same 60,000 dalton peak, plus a 25,000 dalton peak presumably due to the incorporation of DF^{32}P into the active-site containing subunit of plasmin. Therefore, the active plasmin produced when urokinase is incubated with 10% DS migrates as a 98,000 dalton protein on a Sephadex G-150 column and apparently exists as a monomer in solution.

DISCUSSION

The dog plasmin-serum component complex has a molecular weight of 127,000 as determined by polyacrylamide gel electrophoresis and, upon reduction, migrates as a 72,000 dalton species suggesting that the dog serum component complexed with the active-site-containing subunit of plasmin has a molecular weight of 47,000. These results are very similar to those found for the plasmin inhibitor in human plasma (19-21).

In addition to the 127,000 dalton complex observed both in SV3T3 growth media and in urokinase activated plasminogen incubated with dog serum, a 153,000 dalton species is observed in SV3T3 growth medium after three days of incubation. This species is apparently also a

STI and the serum component as demonstrated by the lack of formation of the complex when plasmin is preincubated with STI (Figure 1) before incubation in dog serum and by the lower concentration of plasmin-serum component complex formed when STI is included in the incubation medium.[3] Thus, the dog serum component appears to be indeed an anti-plasmin.

The concentration of the plasmin-antiplasmin complex is observed to decrease with a concomitant increase in the concentration in plasmin which has a proteolytic cut in the active-site containing subunit (degraded plasmin) as the total plasmin concentrations increases. The reduced affinity of the antiplasmin for plasmin which has a proteolytic cut in the active-site-containing subunit is deemed the most likely reason for this decrease in the complex concentration. This degraded plasmin form does not incorporate $DF^{32}P$ (Figure 13) suggesting that the active-site serine is not accessible, and that the degraded plasmin is inhibited. This conclusion is supported by the chromatographic data which indicate that the degraded plasmin migrates with a 212,200 dalton peak and thus is presumably complexed with anti-plasmin or complexed with some other serum component. Whether the cleavage in the active-site subunit of plasmin causes the antiplasmin to act as a noncovalent inhibitor or by the lowered antiplasmin-plasmin binding constant allows other serum inhibitors to become more important cannot be differentiated from these data.

The protease responsible for this cleavage in the active-site subunit of plasmin at high plasmin concentrations remains somewhat of a

mystery. No cell derived material other than the cell plasminogen activator is required for formation of degraded plasmin in serum, for degraded plasmin is formed when plasmin is incubated with serum in the absence of cells. STI inhibits the formation of the degraded plasmin suggesting that the responsible enzyme is trypsin-like in activity. Neither of the trypsin-like enzymes urokinase or plasmin is responsible alone because long incubations of plasminogen with urokinase does not result in the cleavage in plasmin which is observed after only a 30-minute incubation of plasmin with serum (Figure 1). One of the serum enzymes, possibly one of those detected by $DF^{32}P$ incorporation, is apparently responsible for the cleavage. Specifically which enzyme is responsible is not yet known.

Of primary importance to this investigation is whether any active plasmin exists in SV3T3 growth medium, and thus whether plasmin may be the protease responsible for the protease-dependent morphological change exhibited by many neoplastic and virus transformed cells. $DF^{32}P$ inhibitable plasmin is present in the urokinase in situ activation of plasminogen model system designed to mimic SV3T3 growth medium after 48 hours of incubation, implying that active plasmin exists in the cell culture medium. Within the limit of detectibility of the methods used, urokinase and plasminogen do not activate other $DF^{32}P$ inhibitable serine proteases present in dog serum. However, other dog serum components incorporate $DF^{32}P$ at significant levels whether preincubated with urokinase or not. These serum components are probably not responsible for the observed differences in morphology of normal and

transformed cells in culture (13) since both cell types would be exposed to them. Although these results must be considered in light of the fact that the model system used in these investigations assumes that only a plasminogen activator is released by the transformed cells in culture, the implication from the model system is that plasmin is responsible for the protease-dependent morphological change.

ACKNOWLEDGMENTS

We thank Marlyn Teplitz, Maura Kiernan and Lois Kay for their technical assistance. David Agard is to be thanked for his help in the computer graphics used for most of the figures.

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FOOTNOTES

* This work was performed with the support of National Institutes of Health Grant GM-19984 and Grant GM-24485. This paper is Contribution No. 5723 of the Norman W. Church Laboratory of Chemical Biology.

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[1] Abbreviations used: 10% FCS, Dulbecco's modified Eagle's medium with 4.5 times the glucose concentration supplemented with 10% fetal calf serum; Tris buffer: Isotonic Tris buffer (0.025 M Tris, 0.14 M NaCl, 0.005 M KCl, 0.0007 M Na_2HPO_4, 0.0005 M $MgCl_2$, 0.0009 M $CaCl_2$, pH 7.4); 10% DS, Dulbecco's modified Eagle's medium with 4.5 times the glucose concentration supplemented with 10% dog serum; PBS, phosphate buffered saline (0.016 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.137 M

NaCl, 0.0027 M KCl, 0.00049 M $MgCl_2$, 0.0009 M $CaCl_2$, pH 7.4); 3% SDS-BME-Tris buffer, 3% sodium dodecyl sulphate, 0.1 M Tris, 2.5% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, pH 6.8; 3% SDS-Tris buffer, 3% sodium dodecyl sulphate, 0.1 M Tris, 2.5% glycerol, 0.002% bromophenol blue, pH 6.8; STI, soybean trypsin inhibitor; $DF^{32}P$, ^{32}phosphorous-labeled diisopropylfluorphosphate (diisopropylphosphorofluoridate).

[2] Calculation of concentration of plasmin forms: since the three plasmin forms only differ by modifications of the C protein subunit of plasmin, if the relative amounts of the three variations in the C protein are determined, the relative amounts of the three plasmin forms will be likewise determined. These three variations in C protein are: 1) cleavage to 10,000 and 12,000 dalton doublet (D); 2) formation of a complex with the serum component (C-I); and 3) no change (C). This may be expressed as

$$C_T = C + D + C\text{-}I \qquad (1)$$

where C_T is equal to the amount of C protein which would be observed if no modifications had occurred.

From peak integration values of electrophoretograms of reduced and denatured samples (Figures 2 & 8), the quantities C and D are directly determined. The C protein-serum component complex, however, has the same electrophoretic mobility as the B_2 subunit of plasmin thus preventing the direct determination of C-I. This means that

$$B_2^{obs} = B_2 + C\text{-}I \qquad (2)$$

where B_2^{obs} is the area under the peak designated B_2 in the electrophoretograms. Neither of the quantities B_2 or C-I are known, so another independent equation is necessary to evaluate C-I.

It is known that the B_2 protein and the C protein of dog plasmin incorporate iodine in a reproducible manner such that the ratio of the specific activity of the B_2 protein to the C protein is 0.7 (13), thus

$$\frac{B_2}{C_T} = 0.7 \qquad (3)$$

By substituting equations 1 and 2 into equation 3, the quantity C-I is determined and the relative amount of the three forms of plasmin are specified. From the total concentration of plasmin, the concentration of each plasmin form may be determined.

[3] J. Tobler, M. Krieger, and R.M. Stroud, unpublished data.

TABLE I

Comparison of Chromatographic and Electrophoretic Data of SV3T3 Growth Media

The radioactivity in the 98,000 dalton peak on the Sephadex G-150 chromatograms in Figure 4 of 10% DS supplemented with 100 nM ^{125}I-plasminogen and incubated with SV3T3 cells for 24, 48, and 72 hours was determined and expressed as a percent of the total radioactivity of the peaks in each of the chromatograms. The radioactivity in the 94,000 dalton plasminogen peak on the electrophoretograms in Figure 2 of the same samples was determined and expressed as a percent of the total radioactivity of the peaks in each of the electrophoretograms.

Incubation Time Hours	Sephadex G-150 Column % of Total Radioactivity in 98,000 Dalton Peak	Polyacrylamide Gel % of Total Radioactivity in 94,000 Dalton Peak
24	85	78
48	64	61
72	46	45

TABLE II

Distribution of Plasmin Forms Observed in SV3T3 Growth Media

The area under the peaks on the electrophoretograms of reduced and denatured samples of growth media supplemented with 100 nM ^{125}I-plasminogen and incubated for 24, 48, and 72 hours with SV3T3 cells (Figure 2) were integrated and analyzed[2] to determine how the distribution of observed plasmin forms changes with incubation time. The three forms of plasmin, native, degraded, and complexed, refer to plasmin which is in the native form, has a cleavage in the light chain, and is covalently bound to a serum inhibitor, respectively. From the percent of total counts under the plasminogen peak, the percent of plasminogen (initial concentration 100 nM) not activated to plasmin was determined, and thus the concentration of plasmin was determined. The molar concentration of plasmin forms was determined from the percent of total plasmin which each form represented and the total concentration of plasmin.

Incubation Time (Hours)	Distribution of Plasmin Forms (Percent of Total Plasmin)			Concentration of Plasmin Forms (nM)			
	Native	Degraded	Complexed	Native	Degraded	Complexed	Total
24	0.0	0.0	100.0	0.0	0.0	22.1	22.1
48	25.3	22.6	52.1	9.9	8.8	20.4	39.1
72	14.7	62.2	23.1	8.1	34.4	12.8	55.3

TABLE III

Distribution of Plasmin Forms Observed in in situ Urokinase Activation of Plasminogen in 10% DS

The area under the peaks on the electrophoretograms of reduced and denatured 1 ml samples of 10% DS supplemented with 100 nM ^{125}I-plasminogen and incubated with 5, 10, 25, 50, and 100 µl of urokinase (9320 Plough units/ml of PBS) for one hour (Figure 8) were integrated and analyzed[2] to determine how the distribution of observed plasmin forms changes with urokinase concentration. The three forms of plasmin, native, degraded and complexed, refer to plasmin which is in the native form, has a cleavage in the light chain, and is covalently bound to a serum inhibitor, respectively. From the percent of total counts under the plasminogen peak, the percent of plasminogen not activated to plasmin was determined, and thus the concentration of plasmin was determined. The molar concentration of plasmin forms was determined from the percent of total plasmin which each form represented and the total concentration of plasmin.

µl Urokinase Added	Urokinase Conc. (Plough units per ml of 10% DS)	Distribution of Plasmin Forms (Percent of Total Plasmin)			Concentration of Plasmin Forms (nM)			
		Native	Degraded	Complexed	Native	Degraded	Complexed	Total
5	46.6	26.2	21.6	52.2	8.4	6.9	16.8	32.1
10	93.2	22.9	27.3	49.8	8.5	10.1	18.4	36.9
25	233.0	27.7	37.8	34.4	13.9	19.0	17.3	50.3
50	466.0	27.3	52.7	20.0	17.9	34.6	13.1	65.6
100	932.0	29.2	58.5	12.3	23.6	47.2	9.9	80.7

FIGURE LEGENDS

Figure 1: Effect of incubation in 10% DS on electrophoretic mobility of ^{125}I-plasmin. Urokinase activated ^{125}I-plasminogen (a) was incubated with 10% DS (100 nM ^{125}I-plasmin) for 30 minutes (b), and was incubated with 100-fold excess STI for 30 minutes and then incubated with 10% DS for 30 minutes (c). The samples were mixed (1:1) with 3% SDS-BME-Tris buffer, reduced and denatured, and electrophoresed on 10% polyacrylamide gels. The standard curve of log molecular weight as a function of R_F for the electrophoretograms in Figs. 1, 2, and 5 (g).

Figure 2: Electrophoretograms of SV3T3 reduced and denatured growth media. After 24 (a), 48 (b), and 72 (c) hours of incubation, samples of SV3T3 growth media (10% DS) supplemented with 100 nM ^{125}I-plasminogen were mixed (1:1) with the 3% SDS-BME-Tris buffer, reduced and denatured. 10 µl samples were electrophoresed on 10% polyacrylamide gels.

Figure 3: Electrophoretograms of SV3T3 nonreduced growth media. After 24 (a), 48 (b), and 72 (c) hours of incubation, samples of SV3T3 growth media (10% DS) supplemented with 100 nM ^{125}I-plasminogen were mixed (1:1) with the 3% SDS-Trus buffer and denatured. 10 µl samples were electrophoresed on 10% polyacrylamide gels.

Figure 4: Sephadex G-150 chromatograms of SV3T3 growth media. After 24 (——), 48 (– – –), and 72 (▬▬) hours of incubation, 100 µl

samples of SV3T3 growth media (10% DS) supplemented with 100 nM ^{125}I-plasminogen were fractionated on a Sephadex G-150 column equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.9. 7 ml fractions were collected and 100 µl aliquots were counted in a gamma spectrophotometer. The arrows designate the elution volume of blue dextran 2000, aldolase, plasminogen, bovine serum albumin, ovalbumin and tyrosine as observed on independent chromatograms.

Figure 5: Electrophoretograms of reduced and denatured samples of pooled peak fractions of Sephadex G-150 chromatographic separation of radiolabeled species in SV3T3 growth medium. The 212,200 dalton peak fractions (a) and the 98,000 dalton peak fractions (b) of the Sephadex G-150 chromatographic separation of radiolabeled species observed in SV3T3 growth medium (10% DS) supplemented with 100 nM ^{125}I-plasminogen after 72 hours of incubation were pooled, lyophilized, suspended in 3% SDS-BME-Tris buffer, prepared for electrophoresis, and electrophoresed on 10% polyacrylamide gels.

Figure 6: Effects of hydroxylamine incubation on electrophoretic characteristics of SV3T3 nonreduced growth media. After 24 (a,d), 48 (b,e), and 72 (c,f) hours of incubation, samples of SV3T3 growth media (10% DS) supplemented with 100 nM ^{125}I-plasminogen were mixed (1:1) with the 3% SDS-Tris buffer and denatured by immersion in boiling water for 3 minutes. These samples were either mixed (1:1) with 2 M sodium chloride (a,b,c) or mixed (1:1) with 2M hydroxylamine

hydrochloride, pH 7.5 (d,e,f) and incubated two hours at 37°C. The samples were then mixed (1:1) with 3% SDS-Tris buffer and denatured. 25 µl samples were electrophoresed on 10% polyacrylamide gels.

Figure 7: Effects of hydroxylamine incubation on electrophoretic characteristics of SV3T3 reduced and denatured growth media. After 24 (a,d), 48 (b,e) and 72 (c,f) hours of incubation, samples of SV3T3 growth media (10% DS) supplemented with 100 nM ^{125}I-plasminogen were mixed (1:1) with the 3% SDS-BME-Tris buffer and reduced and denatured by immersion in boiling water for three minutes. These samples were either mixed (1:1) with 2M sodium chloride (a,b,c) or mixed (1:1) with 2M hydroxylamine hydrochloride, pH 7.5 (d,e,f) and incubated two hours at 37°C. The samples were then mixed (1:1) with 3% SDS-BME-Tris buffer and reduced and denatured. 25 µl samples were electrophoresed on 10% polyacrylamide gels. The standard curve of log molecular weight as a function of R_F for the electrophoretograms in Figs. 7-10,13,14 (g).

Figure 8: In situ activation of ^{125}I-plasminogen in 10% DS with urokinase. 0 (a), 5 (b), 10 (c), 25 (d), 50 (e), and 100 (f) µl of urokinase (9320 Plough units/ml PBS) were incubated with a 1-ml aliquot of 10% DS supplemented with 100 nM ^{125}I-plasminogen for one hour. The samples were mixed (1:1) with 3% SDS-BME-Tris buffer, reduced, denatured and electrophoresed on 10% polyacrylamide gels.

Figure 9: Effect of preincubation of 10% DS with urokinase on DF^{32}P incorporation into serum components. A 2-ml aliquot of 10% DS

supplemented with 100 nM dog plasminogen was either incubated for one hour with 93.2 Plough units of urokinase/ml (▬▬), or incubated for one hour without urokinase addition (——). 3.3 mg of $DF^{32}P$ in 2-propanol was added (final $DF^{32}P$ concentration 8.2 mM) and incubated for one hour with constant stirring followed by dialysis overnight. Reduced samples were electrophoresed on 10% polyacrylamide gels.

Figure 10: Electrophoretograms of $DF^{32}P$-labeled urokinase and plasmin. A 2-ml aliquot of a 100 nM plasminogen solution in PBS was incubated with 20 µl of urokinase (9320 Plough units/ml) for one hour. 100 µl of $DF^{32}P$ solution (16.6 mg $DF^{32}P$/ml of 2-propanol) was incubated at room temperature for one hour with constant stirring, and dialyzed overnight. Reduced (a) and nonreduced (b) samples were electrophoresed on 10% polyacrylamide gels.

Figure 11: Effect of preincubation of 10% DS with urokinase on $DF^{32}P$ incorporation into serum components. A 2-ml aliquot of 10% DS supplemented with 100 nM dog plasminogen was either incubated for one hour with 93.2 Plough units of urokinase/ml (▬▬) or incubated for one hour without urokinase addition (——). 3.3 mg of $DF^{32}P$ in 2-propanol was added (final $DF^{32}P$ concentration 8.2 mM) and incubated for one hour with constant stirring followed by dialysis overnight. Nonreduced samples were electrophoresed on 10% polyacrylamide gels.

Figure 12: Sephadex G-150 chromatographic characterization of plasminogen species present in DF^{32}P incubation. A 2-ml aliquot of 10% DS was treated identically to that used for the DF^{32}P-labeling experiment except that it was supplemented with 100 nM ^{125}I-plasminogen to monitor the effects of the one-hour incubation of 20 µl of urokinase (9320 Plough units/ml PBS) on plasminogen. A 100-µl aliquot was fractionated on a Sephadex G-150 column equilibrated with 0.1 M ammonium bicarbonate buffer, pH 7.9. 7 ml fractions were collected and 50 µl aliquots were counted in a gamma spectrophotometer.

Figure 13: Electrophoretograms of serum components which incorporated DF^{32}P and eluted from a Sephadex G-150 column with those fractions comprising the 212,200 dalton plasmin-containing peak. Samples of 10% DS incubated with DF^{32}P both with and without preincubation with urokinase were fractionated on a Sephadex G-150 column and those fractions known to comprise the 212,200 dalton plasmin-containing peak were pooled, lyophilized, prepared for electrophoresis and electrophoresed on 10% polyacrylamide gels. Only one of each of the reduced (a) and nonreduced (b) electrophoretograms is presented because samples which were preincubated with urokinase were virtually identical with those not preincubated with urokinase.

Figure 14: Electrophoretograms of serum components which incorporated DF^{32}P and elute from a Sephadex G-150 column with those factions comprising the 98,000 dalton plasminogen-containing peak.

Samples of 10% DS incubated with DF ^{32}P both without (a) and with (b) preincubation with urokinase were fractionated on a Sephadex G-150 column and those fractions known to comprise the 98,000 dalton plasminogen-containing peak were pooled, lyophilized, reduced and denatured and electrophoresed on 10% polyacrylamide gels.

Figure 1

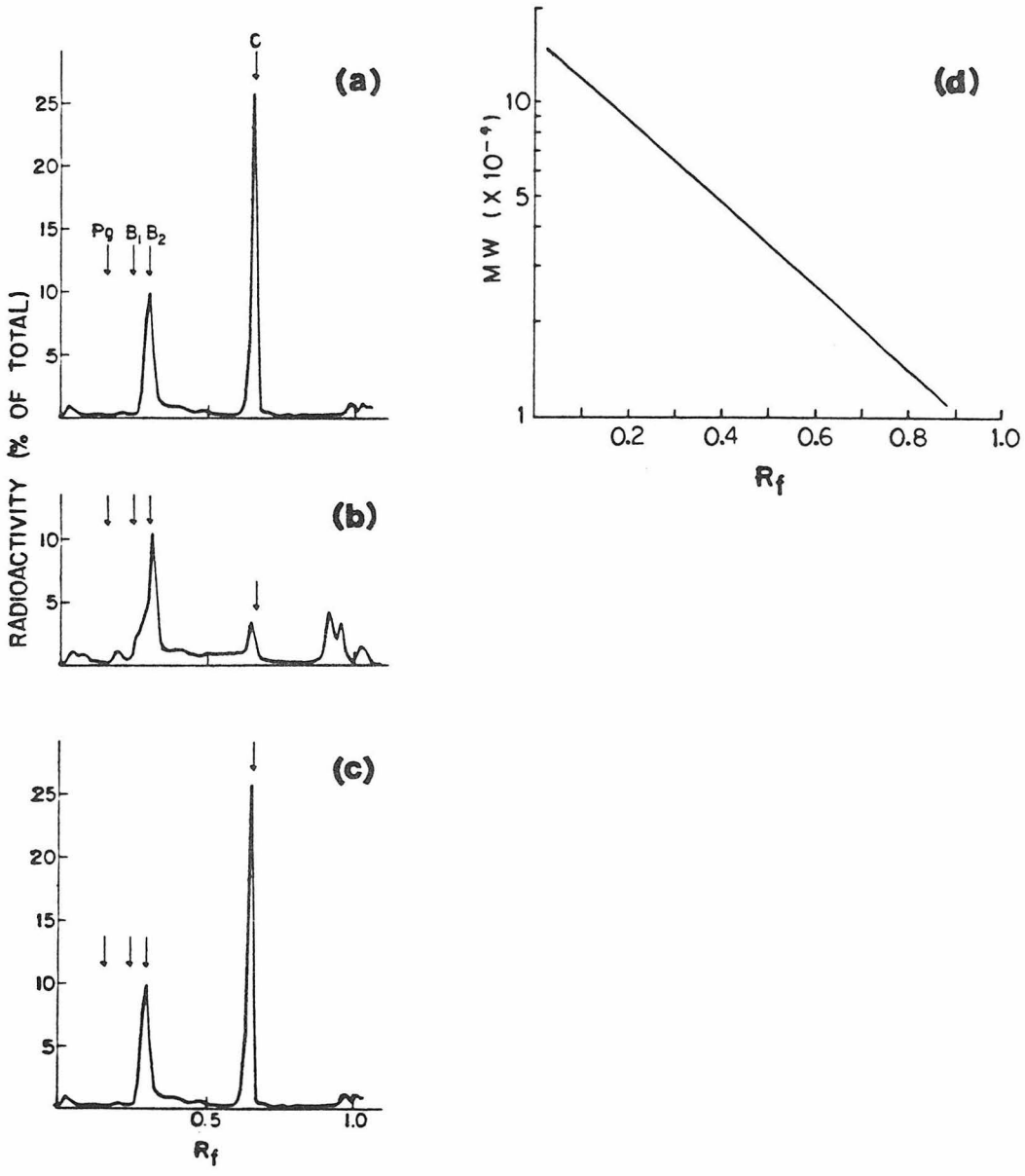

(a)
C
Po
B1
B2
25
20
15
10
5
RADIOACTIVITY (% OF TOTAL)
(b)
10
5
(c)
25
20
15
10
5
0.5
1.0
Rf
(d)
MW (X 10⁻⁴)
10
5
1
0.2
0.4
0.6
0.8
1.0
Rf

Figure 2

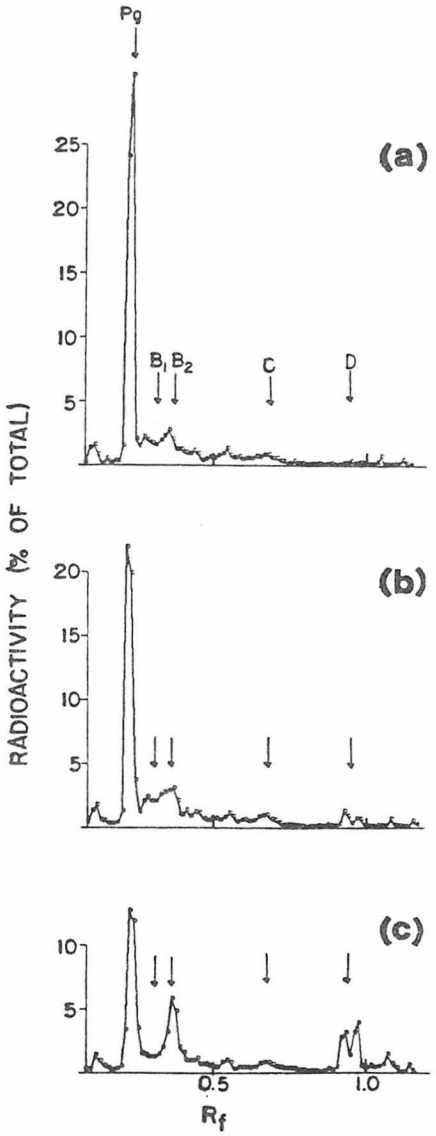

Pg
(a)
25
20
15
10
5
B1 B2
C
D
RADIOACTIVITY (% OF TOTAL)
(b)
20
15
10
5
(c)
10
5
0.5
1.0
Rf

Figure 3

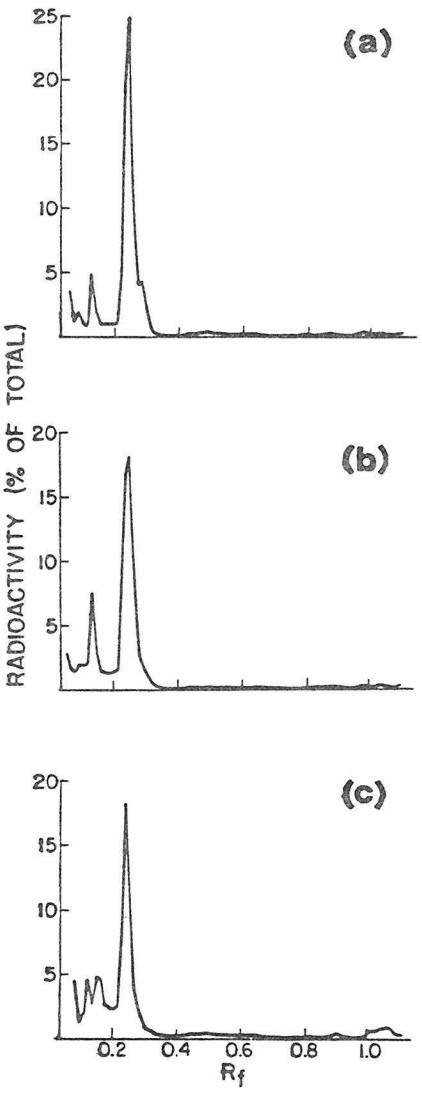


Figure 4

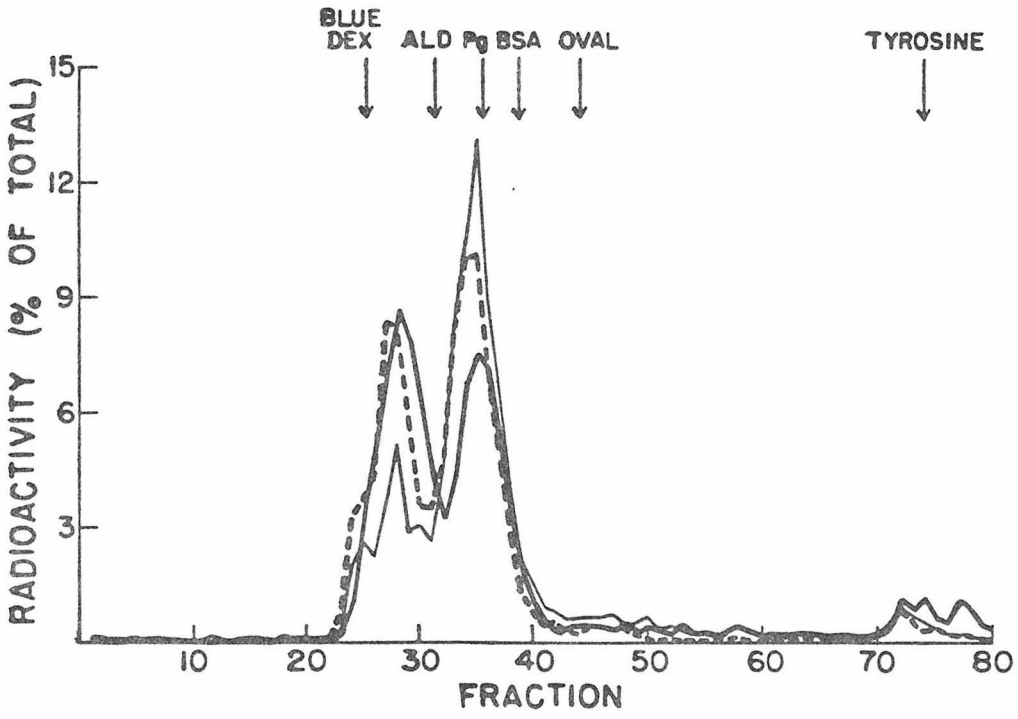
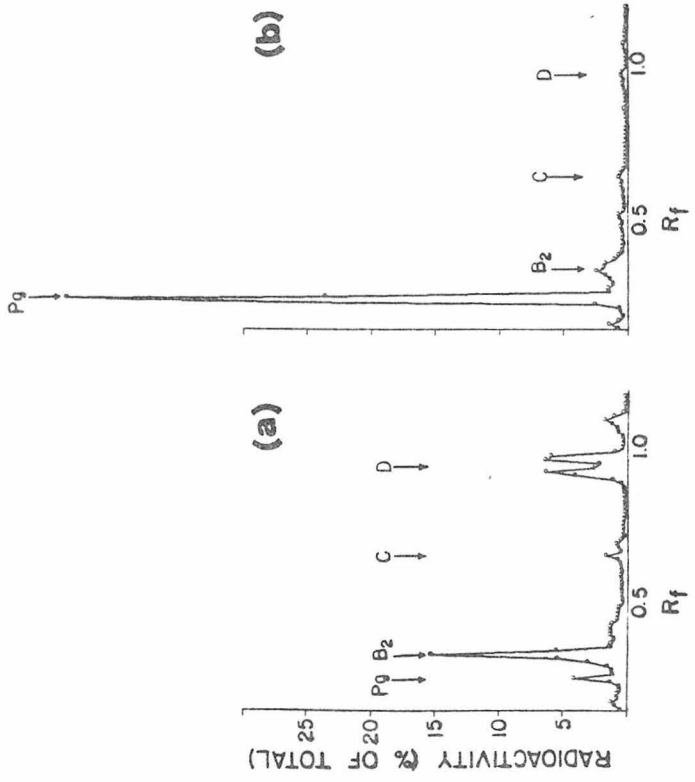

BLUE DEX
ALD
Pg
BSA
OVAL
TYROSINE
RADIOACTIVITY (% OF TOTAL)
15
12
9
6
3
10
20
30
40
50
60
70
80
FRACTION

Figure 5



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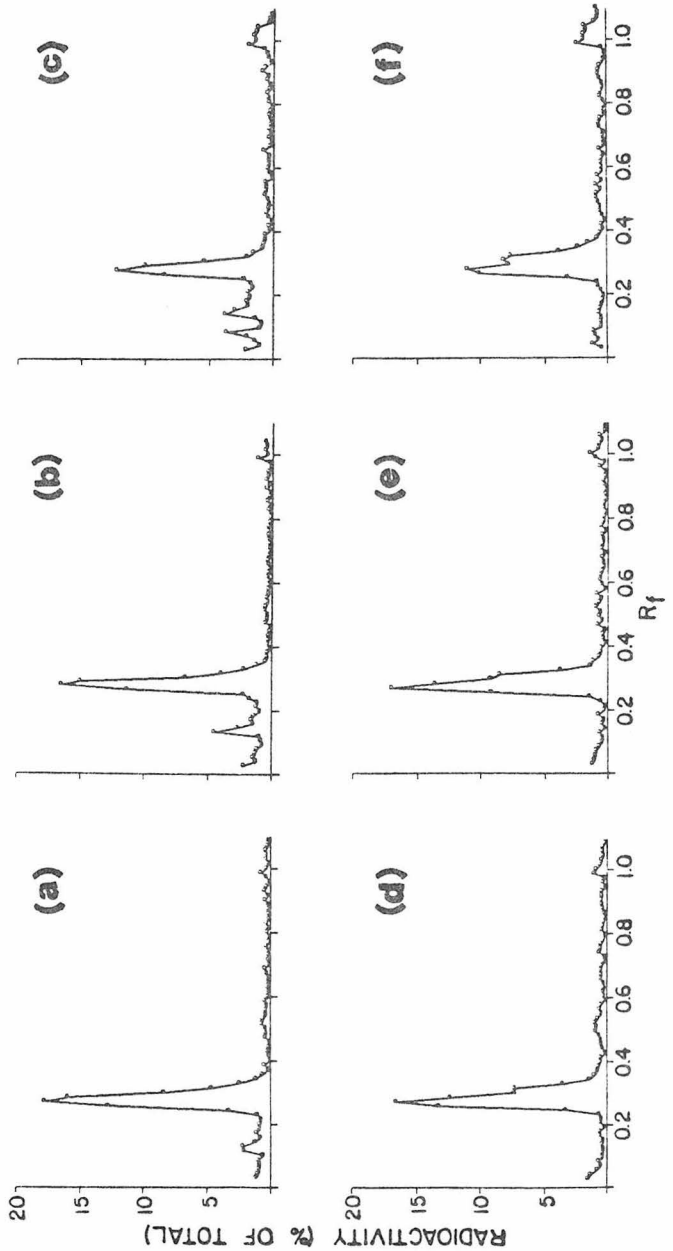

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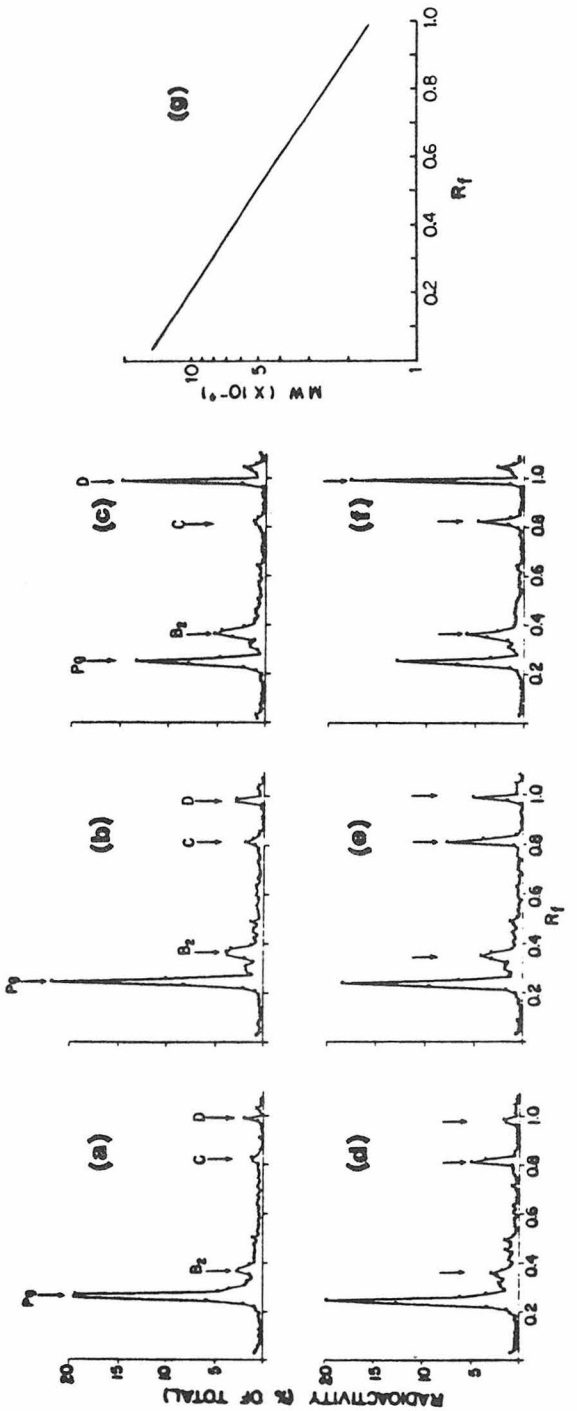


Figure 8

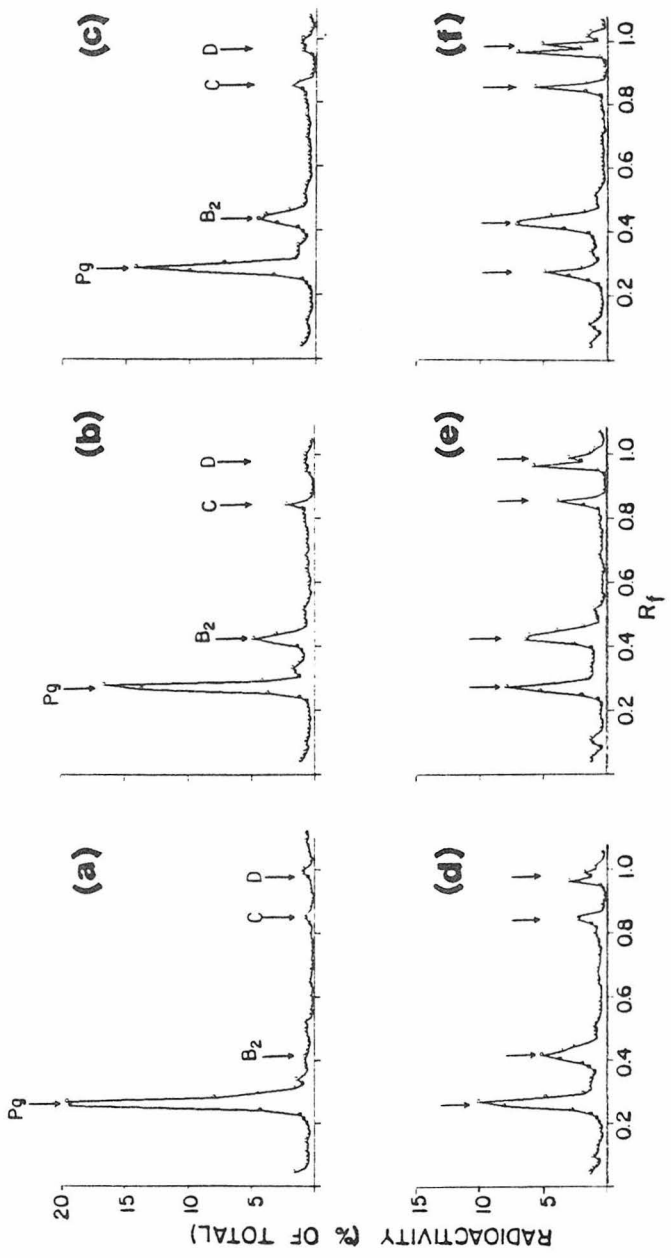

(a)
(b)
(c)
(d)
(e)
(f)
Pg
B2
C
D
RADIOACTIVITY (% OF TOTAL)
Rf
0.2
0.4
0.6
0.8
1.0
5
10
15
20

Figure 9

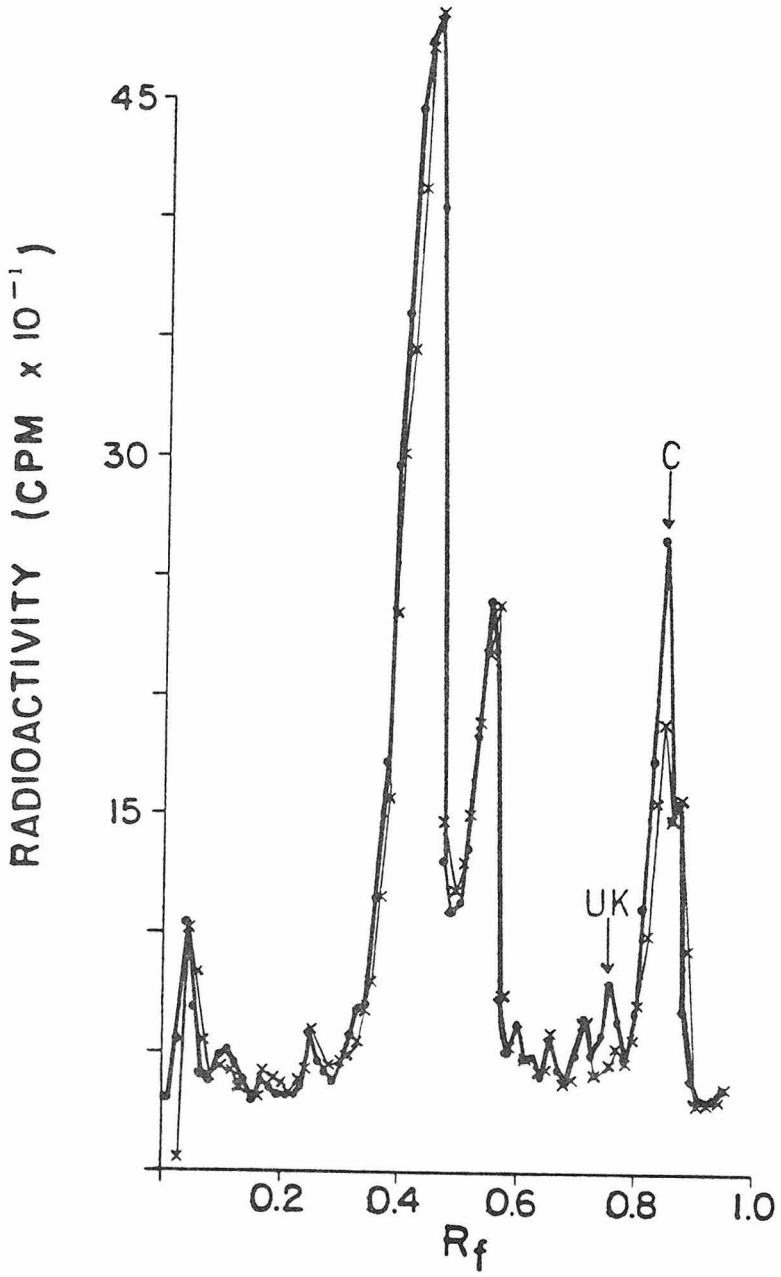


Figure 10

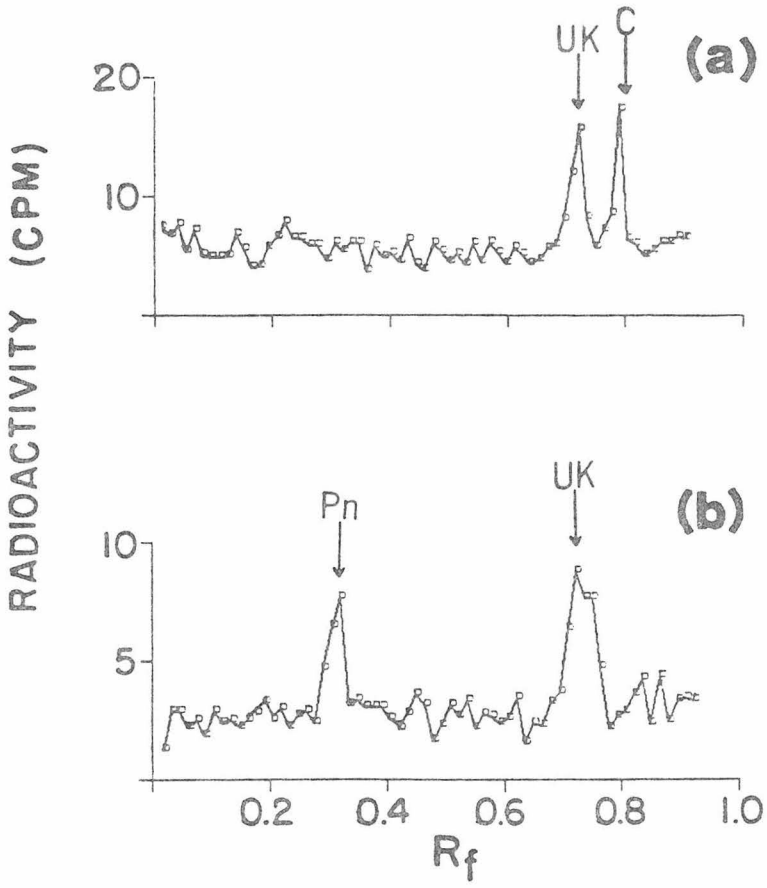


Figure 11

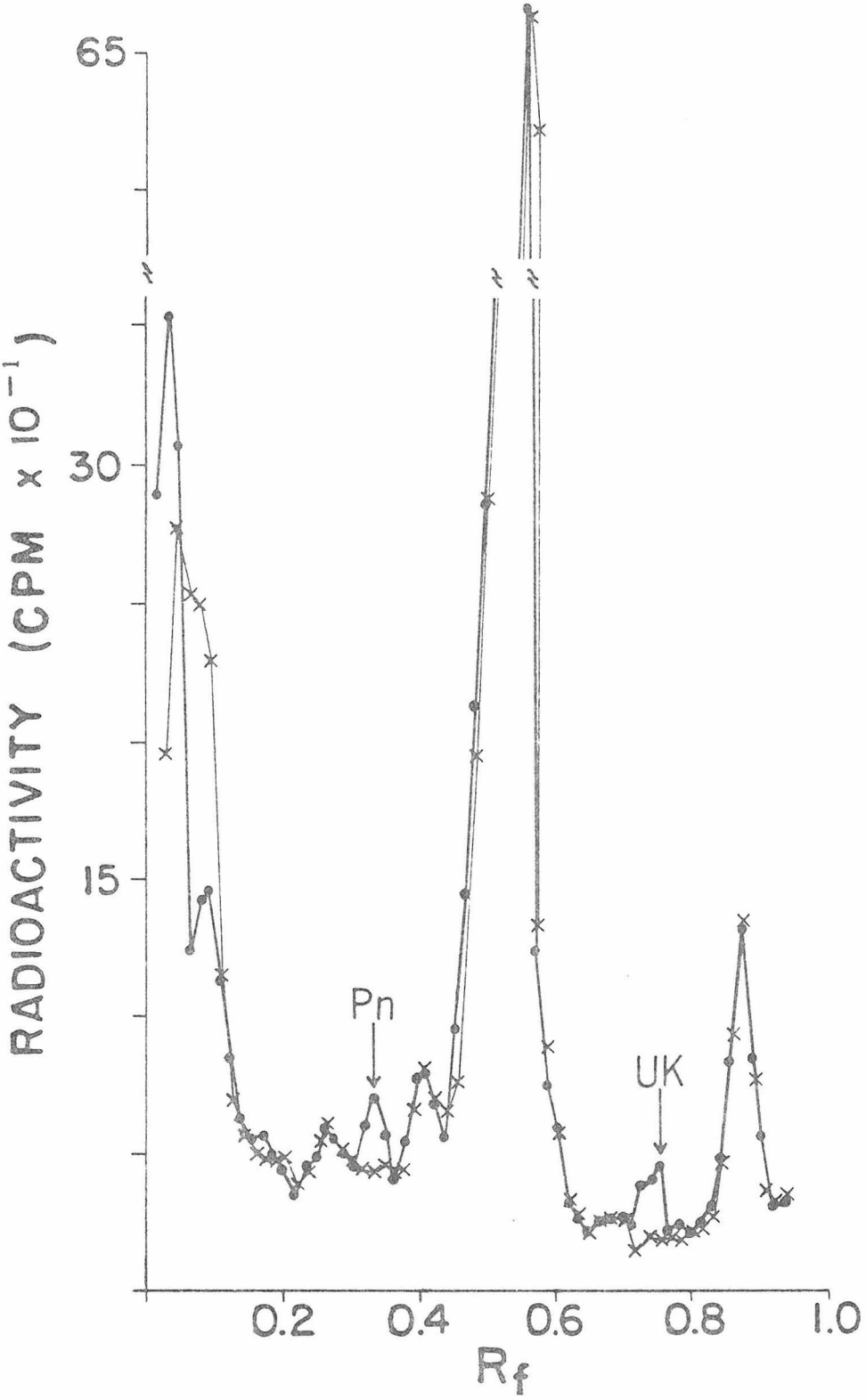


Figure 12

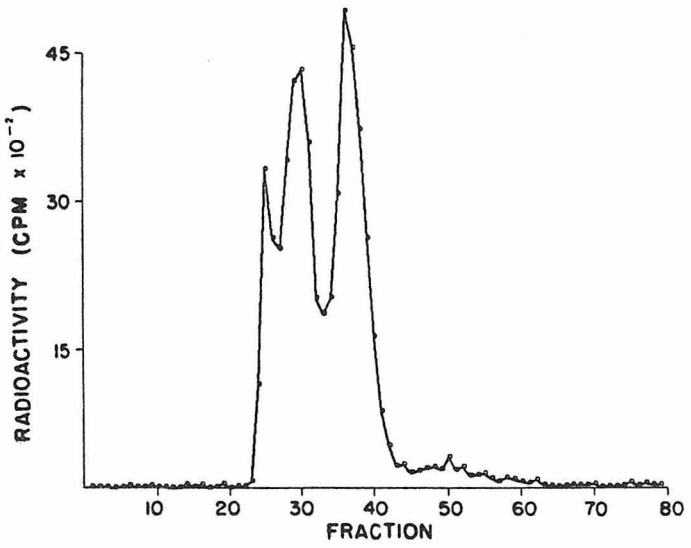

45
30
15
RADIOACTIVITY (CPM x 10^{-2})
10
20
30
40
50
60
70
80
FRACTION

Figure 13

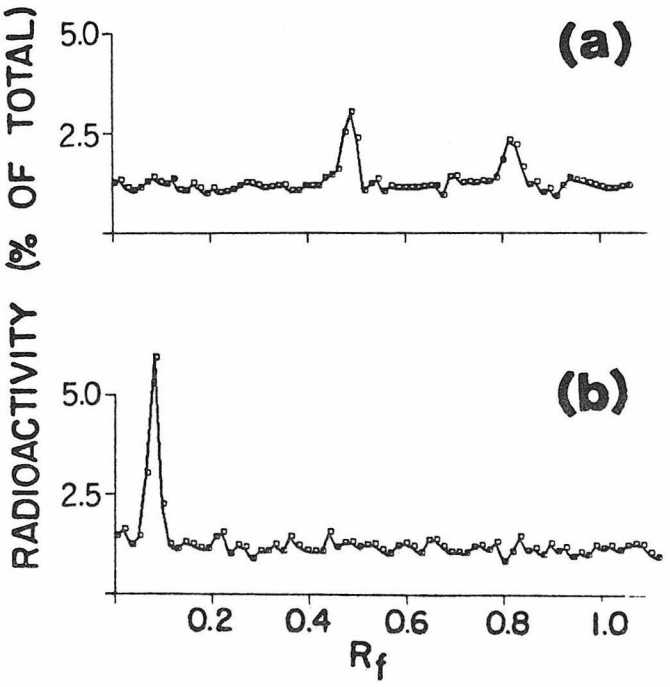
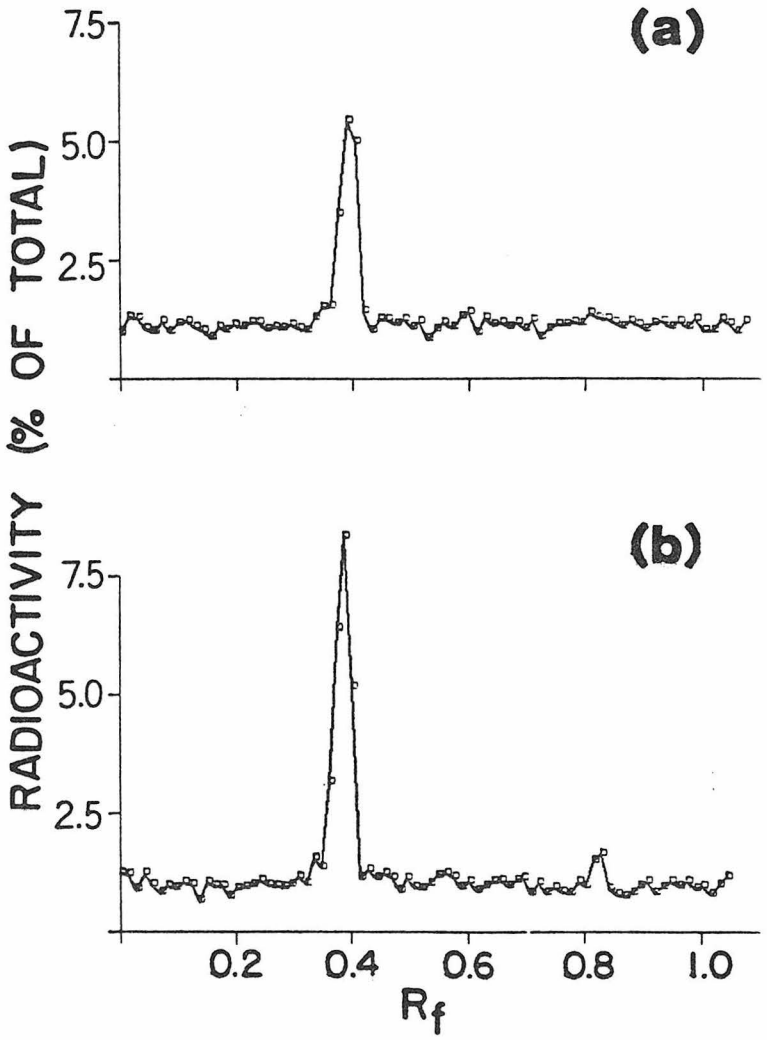


Figure 14



APPENDIX III

ANALYTICAL BIOCHEMISTRY 81, 450–453 (1977)

Micro-Step-Exclusion Chromatography

The rapid method for desalting small volumes of liquid developed by Neal and Florini [(1973) *Anal. Biochem.* **55**, 328–330] can easily be extended to permit rapid column chromatographic separations. Micro-step-exclusion chromatography has been used to separate and identify monoiodotyrosine as the radiolabeled low molecular weight degradation product of ^{125}I-labeled plasminogen, formed when plasminogen binds to Balb/c SV 3T3 cells. This method has also been used to monitor the time course of the degradation.

Neal and Florini (1) have shown that small volumes of solutions can be rapidly desalted using a small column of Sephadex G-25 and a clinical centrifuge. In their procedure, the Sephadex G-25 is packed into a centrifuge tube which has a filter paper-covered hole at the bottom, and the void volume of liquid is removed by centrifugation. A sample layered on the column can then be desalted by a second centrifugation, during which the macromolecules pass through the column into a collecting tube, and the sample buffer is exchanged for the buffer previously equilibrated with the column.

We have extended this procedure by performing additional elution–centrifugation steps in order to obtain a complete step chromatogram of the sample. This simple procedure, micro-step-exclusion chromatography, separates molecules much more rapidly than does standard column chromatography. Each chromatographic step takes 2–3 min, and a five-step chromatogram can resolve a sample (see below). An additional advantage of this technique is that, since liquid in the void volume is removed at each step, sample dilution is minimized. This can be particularly important when small ($<50\ \mu$l) relatively dilute samples must be chromatographed.

To obtain a chromatogram, a sample is layered on top of the column-packing material in a microcentrifuge tube which is then inserted into a larger conical centrifuge tube, and the apparatus (Fig. 1) is spun for 1 or 2 min in a clinical centrifuge at approximately 2500 rpm. The lip on the microcentrifuge tube and the taper of the outer tube keep the microcentrifuge tube above the level of the column eluate when the liquid reaches the bottom of the outer tube. Portions of the sample remaining in the column can be eluted in steps by layering on additional buffer, transferring the microcentrifuge tube to another outer conical tube, and repeating the centrifugation.

We have used this technique to separate and identify monoiodotyrosine

ISSN 0003-2697

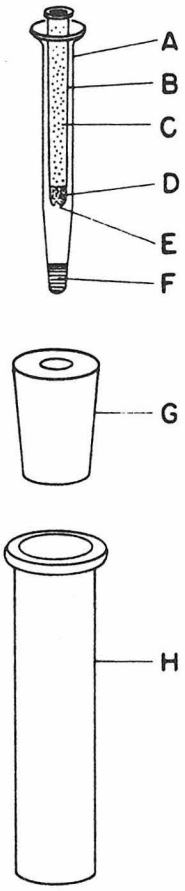


FIG. 1. Apparatus for micro-step-exclusion chromatography: (A) 1-ml outer conical centrifuge tube; (B) 3.5-cm-long, ~400-μl-capacity, inner conical polyethylene microcentrifuge tube; (C) ~300 μl column packing material (Bio-Gel P-2); (D) glass-wool plug; (E) 0.8-mm-diameter hole; (F) column eluate; (G) No. 2 rubber stopper with ~0.8-cm-diameter hole; (H) clinical centrifuge bucket. The lip on the polyethylene tube and the taper of the outer tube keep the microcentrifuge tube above the level of the eluate.

as the radiolabeled low molecular weight degradation product of ^{125}I-labeled plasminogen, formed when plasminogen binds to Balb/c SV 3T3 cells, and to monitor the time course of the degradation. After being incubated with ^{125}I-labeled plasminogen, the cells were extensively washed and then completely dissolved in an SDS–Tris buffer (3% SDS, 0.1 M Tris, 2.5% glycerol, 5% β-mercaptoethanol, 0.002% bromophenol blue, pH 6.8) to prepare the samples for chromatography. The samples were also immersed in boiling water for 3 min and were vigorously passed through Pasteur pipets to reduce viscosity.

Using 25- and 50-μl samples and 50-, 100-, and 200-μl elution steps,

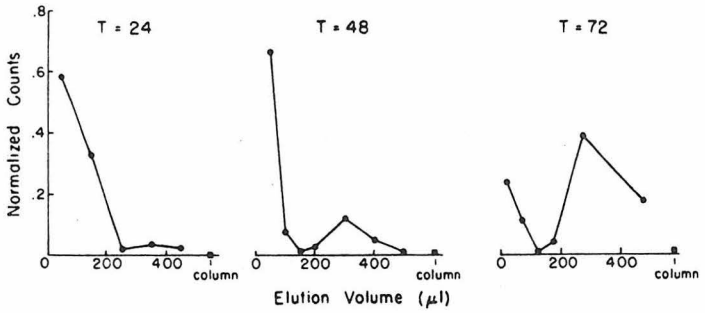


FIG. 2. Micro-step-exclusion chromatograms. ^{125}I-Labeled plasminogen (~100 nM) was incubated with Balb/c SV 3T3 cells in Dulbecco's modified Eagle's medium with 4.5-fold the glucose concentration and supplemented with 10% dog serum. At 24-hr intervals, the cells were scraped from the petri dishes, washed extensively, dissolved in an SDS–Tris buffer, and subjected to micro-step-exclusion chromatography on Bio-Gel P-2 equilibrated with a 3% SDS, 0.1 M Tris buffer, pH 6.8. The eluate fractions were counted on a gamma spectrometer, and the counts in each fraction were normalized by the total counts in each sample (2059 cpm in 50 μl at T = 24 hr; 3405 cpm in 50 μl at T = 48 hr; 4793 cpm in 25 μl at T = 72 hr). The amounts of radioactivity remaining in each of the columns after chromatography were determined, and the values are included in the chromatograms. The chromatograms show that the proportion of radiolabeled low molecular weight degradation product (monoiodotyrosine) increased with time, while the proportion of labeled macromolecules decreased with time.

the lower molecular weight components of the SDS–cell solutions were clearly resolved from the larger molecules in five- to seven-step chromatograms (Fig. 2). The bromophenol blue in the sample buffer was used to monitor the position of low molecular weight material during chromatography. For the 48- and 72-hr samples, small eluant volumes (50 μl) were employed in the first steps of chromatography to obtain high-resolution separations, after which larger volumes were used to minimize the total number of steps required to elute the sample completely.

The low molecular weight fraction was further analyzed by ascending paper chromatography [butanol:acetic acid:water (4:1:2, v/v)] (2). Standard samples of monoiodotyrosine (3-iodo-L-tyrosine, Aldrich Lot 082837) and diiodotyrosine (3,5-diiodo-L-tyrosine, Aldrich Lot 071637) were co-chromatographed with this sample and were visualized with uv light and ninhydrin spray staining [0.2% in acetone (v/v)]. The radiolabeled molecules in this low molecular weight fraction co-migrated with monoiodotyrosine during the paper chromatography. These results demonstrate that micro-step-exclusion chromatography can rapidly and efficiently separate the low molecular weight products of cellular protein degradation from macromolecules. In this case, the proportion of the cell-associated degradation product, ^{125}I-labeled monoiodotyrosine, increased with time, while the proportion of radiolabeled macromolecules decreased with time. A detailed account of the binding and processing

of plasminogen by Balb/c 3T3 and SV 3T3 cells will be published elsewhere (3).

In addition to being a rapid method of monitoring the kinetics of degradation of plasma membrane proteins, micro-step-exclusion chromatography should be applicable to ion-exchange and affinity chromatography and other types of gel-filtration chromatography, provided that the packing material can withstand the centripetal force developed during centrifugation. This simple rapid procedure should also be valuable in developing protein purification protocols on a small scale.

ACKNOWLEDGMENTS

We thank Dr. Ray Owen for the use of the gamma spectrometer and Dr. Robert Stroud for his constant support and valuable advice. This work was supported by National Institutes of Health Grant No. GM-19984. One of us (M.K.) was the recipient of a Danforth Foundation Fellowship, and another (J.T.) was recipient of a National Institutes of Health Predoctoral Traineeship. This is contribution No. 5474 of the Norman W. Church Laboratory of Chemical Biology.

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3. Tobler, J., Krieger, M., and Stroud, R. M., manuscript in preparation.

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Received January 10, 1977; accepted April 14, 1977

PROPOSITION I

INTRODUCTION

Objective

The increased fibrinolytic (proteolytic) activity associated with virally-transformed cells in culture (1-4) may attenuate plasma membrane glycosyltransferase activity and thus indirectly cause alteration in glycoproteins and glycolipids observed on transformed-cell membrane surfaces. Exogenous protein and lipid sugar acceptors and labeled sugar-nucleotides are proposed for measurement of the cell surface glycosyltransferase activity in synchronous normal and virally-transformed fibroblasts. Macromolecular protease inhibitors in concentrations great enough to prevent fibrinolytic activity as detected by fibrin plate assays will allow measurement of the magnitude of the proposed attenuation of glycosyltransferase activity.

Background

Plasma Membrane Glycosyltransferase Activity

Cell surface glycosyltransferase activity has been found on cells of many types including: human platelets (5), chicken embryo neural retina cells (6), chick embryo cells (7), neonatal rat lymphocytes (8), rat intestinal epithelium cells (9), and DNA- and RNA-virus transformed or nontransformed fibroblasts (10-14).

Of particular interest are the experiments comparing cell surface transferase activity of transformed and nontransformed cells. However, none of the published experiments allow measurement of the magnitude of enzyme activity on the cell surface and a comparison of glycosyltransferase activity of transformed and untransformed cells under similar growth conditions. The experiments were designed such that: 1) transferase activity of log phase transformed cells was compared with confluent nontransformed cells (10,14); 2) endogenous sugar acceptors were used which may be limiting and thus total enzyme activity is not measured (11,12); and 3) trypsin was used to harvest cells which may inactivate some of the cell surface glycosyltransferases (13).

Glycolipid: Glycosyltransferase and Glycolipid Alterations with Viral Transformation

Using cell homogenates (and thus the enzyme activity associated with the Golgi apparatus, the mitochondrion, and the plasma membrane is measured) with purified glycolipid acceptors and labeled sugar-nucleotides, the glycolipid:glycosyltransferase activity of established transformed and nontransformed mouse (15-17) and hamster (18, 19) cell lines has been determined. In general, a reduction of enzyme activity was associated with the transformed cells compared with the parent cells.

This lowered glycolipid:glycosyltransferase activity in transformed cells is manifested by a reduction in the number of "higher"

(greater than two sugar groups) gangliosides observed on the cell surface. A reduction of higher gangliosides has been found in mouse (20-27), hamster (20,28-31), human (29), and chicken (32) cells transformed by DNA and RNA viruses.

Glycoprotein:Glycosyltransferases and Glycoprotein Alterations with Viral Transformation

Using cell homogenates with purified glycoprotein acceptors and labeled sugar-nucleotides, the glycoprotein:glycosyltransferase activity of transformed and nontransformed 3T3 cells has been determined. Grimes has found a reduction in transferase activity (33), while Bosmann has found an increase in activity (34,35) with viral transformation.

The effect of transformation on cell membrane glycoproteins is similarly ambiguous. Less glycosylation has been found by Ohta et al. (36) and Chiarugi et al. (37). In contrast, van Beek et al. (38), Glick et al. (39), and Warren et al (40,41) have found greater glycosylation in transformed cell membranes. One must keep in mind that protein alterations have been observed in transformed cell membranes (42-44) such that the differences in glycosylation may be due to differences in acceptor molecules. Many of the assays (38-41) for glycosylation involve trypsinization of the glycopeptides from the cell surface and thus depend upon availability of these molecules. Topological differences in the cell membranes of different clones, or from viral transformation could have an effect on the observed results.

Rationale

A repressor-like mechanism has been proposed to explain the differences in glycosylation of transformed cells by some authors (22,24). In light of the increased proteolytic activity associated with transformed cells and the existence of plasma membrane glycosyltransferases, an alternative explanation is that: 1) the terminal sugar groups of glycolipids and glycoproteins are added by plasma membrane glycosyltransferases; 2) high proteolytic activity brought about by viral transformation attenuates the transferase activity through degradation of these enzymes and causes a reduction in glycosylation.

The effects of proteolytic activity on glycosylation may be tested using exogenous acceptors and labeled sugar donors to measure plasma membrane glycosyltransferase activity of transformed and untransformed cells both in the presence and absence of a protease inhibitor.

Synchronous cell populations will be used for the study because Bosmann (45) has observed variations in glycosyltransferase activity with the cell cycle. Primary cultures will be used to avoid the problem of high fibrinolytic activity associated with nontransformed cells which have been extensively serially passaged in culture (46). Excess exogenous glycoprotein and glycolipid with labeled sugar-nucleotides will be used in the assay so that the total enzyme activity is measured. To inhibit the proteolytic activity, macromolecular protease inhibitors (bovine pancreatic trypsin inhibitor or soybean trypsin inhibitor) will be used. Small molecule inhibitors have been

shown to effect other cell systems (47).

METHODS OF PROCEDURE

Cell Synchrony

The physical selection technique of Terasima and Tolmach will be used (48). This method is based on the fact that cells in mitosis are rounded and less adherent to their substratum and thus more easily dislodged from the surface than interphase cells.

Preparation of Acceptors

Glycoprotein acceptors will be prepared from fetuin and porcine submaxillary glycoprotein treated with neuraminidase to remove sialic acid, and/or β-galactosidase to remove galactose (34).

N-acetylneuraminylgalactosylglucosylceramide (GM_3) will be purified from dog erythrocytes (23). N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosylceramide (GM_2) will be prepared from the brain of a patient with Tay-Sachs disease (49). Galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosylglucosylceramide (GM_1) will be prepared from bovine brain gangliosides (50).

Glycosyltransferase Assay

Glycoprotein:Glycosyltransferase

Four cell groups will be assayed: 1) nontransformed; 2) transformed; 3) nontransformed grown with PTI (pancreatic trypsin inhibitor); and 4) transformed grown with PTI. The cells will be harvested at various points in the cell cycle using EDTA to remove the cells from the substratum. The cells will be suspended in Tris buffer with excess ^{14}C-labeled precursor and exogenous acceptor. Four assays will be made: 1) fetuin minus sialic acid plus CMP-^{14}C-N-acetylneuraminic acid (CMP-NANA); 2) fetuin minus sialic acid and galactose plus UDP-^{14}C-galactose; 3) porcine submaxillary glycoprotein (PSG) minus sialic acid plus CMP-^{14}C-NANA; and 4) PSG minus sialic acid and galactose plus UDP-^{14}C-galactose. Following the method of Bosmann (10) after the incubation and removal of cells, 1% phosphotungstic acid in 0.5 N HCl will be used to precipitate the protein and the specific activity of the pellet will be determined.

Glycolipid:Glycosyltransferase

The cells will be prepared for the assays as described above. Three assays will be made: 1) GM_3 plus UDP-^{14}C-N-acetylgalactosamine; 2) GM_2 plus UDP-^{14}C-galactose; and 3) GM_1 plus CMP-^{14}C-NANA. Following the procedure of Fishman et al. (15) after the incubation and removal of the cells, the reactions will be terminated by adding 20 volumes of chloroform-methanol (2:1 V/V) and 40 volumes of chloroform-

methanol-water (60:30:4.5 V/V/V) and the mixture passed through a Sephadex G-25 equilibrated with chloroform-methanol-water to separate the radioactive glycolipid from the precursor.

Proteolytic Activity

Fibrin plate assays of cell supernatants will be used to determine how much PTI is necessary to inhibit the proteolytic activity (3).

SIGNIFICANCE

Viral transformation of cells in culture is accompanied by cell membrane alterations including changes in agglutinability properties by lectins, changes in glycolipids and glycoproteins, increase in proteolytic activity, increase in sugar transport, and decrease in cAMP levels. Presently, the interrelationship, if one exists, among these phenomena is not know. Through understanding of these membrane changes, insight may be gained to the mechanism of viral transformation and the phenotypic changes which accompany it.

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PROPOSITION II

INTRODUCTION

Objective

An *in utero* contraceptive device is proposed which requires the presence of semen in the uterus for the release of a drug which prevents fertilization by inhibiting sperm penetration of the zona pellucida of the ovum.

Background

Drugs which interfere with the reproductive process may be categorized into several main classes: 1) drugs administered to the male to control spermatogenesis; 2) vaginal spermicidal agents to inhibit released spermatozoa; 3) drugs administered to females to suppress ovulation or inhibit corpus luteum formation; and 4) drugs administered to females to inhibit implantation or to destroy blastocysts.

Experience with such agents has shown the necessity for concern over problems of safety following long-term usage. Side effects are a continuing hazard with many of the drugs now in use, and in some cases the inhibition of reproductive potential is unalterable. Moreover, there is the very important factor of cost to the user. Finally, there is the recognition that the relative merit of any drug is measured by consumer acceptance and the ability of the user to

follow instructions. To date no one has developed a side-effect free, inexpensive, easy to use contraceptive which is attractive to the consumer.

By changing the emphasis of contraceptive design from inhibition of ovagenesis and spermatogenesis (i.e., inducing temporary sterility) to inhibition of fertilization of the ovum, the side effects which now plague hormonal contraceptives may be avoided. The available alternatives to hormonal contraceptives are intrauterine contraceptive devices (IUCD), topical agents (spermicidal creams and foams), and containment devices (condoms and diaphrams). IUCD's are dangerous because of the possibility of perforation of the uterus, and their use is often accompanied with cramping and heavy bleeding both during menses and inter-menses. The remaining techniques, although essentially side-effect free, are inconvenient to use and are not among the most popular contraceptive methods for this reason. However, by making topical agents more convenient to use, one would have a contraceptive which meets the criteria outlined above. One approach would be to use a device which relies on the biochemical properties of seminal plasma for the release of a contraceptive drug. This approach will be discussed in detail.

Four proteases have been reported in seminal plasma: pepsinogen, seminin (a neutral protease), plasmin(ogen), and seminal plasminogen activator (1-4). The presence of plasmin(ogen) is in doubt and has only been found by a few authors and in extremely small quantities (1,4). Protease inhibitors also are found in seminal plasma (5-10)

but a net fibrinolytic activity is expressed (11) and is thought to be due to the plasminogen activator (12,13). The seminal plasminogen activator has been isolated and characterized (13,14) and shown to be immunologically identical with urokinase (15), a plasminogen activator found in the urine (16) and in many tissues throughout the body (17). Although it has not yet been proven, the seminal plasminogen activator probably activates plasminogen by a highly specific cleavage of an arginine-valine bond as is known to be the case for urokinase activation of plasminogen (18-20). Thus, if a drug were attached to a solid support by an arginine-valine linkage, the drug would only be released when seminal plasma and thus seminal plasminogen activator was present. The solid support could be a nylon tube which would be placed in the uterus thus allowing a convenient administration of a topical contraceptive drug.

If plasminogen activator activity or inhibitors of this activity were normally present in the uterus, many of the advantages of this contraceptive device would be negated. The cells of the inner lining of the uterus which make up the endometrium have been shown to have increasing concentrations of plasminogen activator as the menstrual cycle progresses, reaching a maximum just before menstruation (21-24). Presumably these cells release the plasminogen activator as menstruation begins (21). However, no systematic study of the plasminogen activator activity in the human uterus cavity has been undertaken.

Cervical mucus has been found to contain five protease inhibitors: α_1-antitrypsin, inter-α-trypsin inhibitor, α_1-antichymotrypsin,

antithrombin III and C'_1-esterase inactivator (25). These protease inhibitors have their lowest concentration during the fertile period of the cycle when cervical mucus is most receptive for sperm penetration (26,27). Rhesus monkey oviduct fluid proteinase inhibitor concentrations follow the same cyclical pattern observed for human cervical mucus (28) suggesting that the proteinase inhibitor concentrations of the entire female genital tract is very low at midcycle. Thus in terms of the proposed contraceptive device, when it is most important that proteolytic activity is expressed, the protease inhibitor concentrations of the female genital tract are lowest. The relatively high concentrations during other portions of the cycle are also advantages for these inhibitors may suppress any plasminogen activator activity of the uterus.

Other characteristics of the female genital tract are advantageous to the proposed contraceptive device. Because of cyclical changes in the viscosity and other properties of cervical mucus, sperm and semen transport through the cervical os only occurs during the late proliferative and ovulatory portions of the menstrual cycle (29,30). The seminal plasminogen activator will only be present in the uterus during the fertile portion of the cycle, therefore, the contraceptive drug will only be released when conception is possible and not released during the infertile portions of the cycle. Even when sperm can be transported through the cervical os, only about 7% of the inseminated sperm make their way to the oviducts (31). Thus, the number of sperm to be dealt with is significantly less than when topical agents are

used in the vagina. In addition, the drug is being released closer to the point of conception so that transport of the drug is not as great a problem.

Drugs which may be attached to the proposed contraceptive device fall into two classes: 1) agents which disrupt spermatozoa membranes, and 2) agents which prevent spermatozoa penetration of the ovum. Disruptive agents on the market today are all derivatives of poly(ethylene glycol)p-phenyl ether. Agents which prevent spermatozoa penetration of the ovum include synthetic, low molecular weight, irreversible protease inhibitors (32-35). For rabbits, hamsters, and deer, synthetic protease inhibitors effectively prevent fertilization if added to capacitated or ejaculated sperm or if deposited into the vagina before coitus (32-35). Furthermore, the addition of such synthetic inhibitors to vaginal contraceptive creams greatly increased the antifertility properties of these creams. Presumably, these inhibitors are acting upon acrosin, the protease found in the acrosome of spermatozoa which is thought to be necessary for sperm penetration of the zona pellucida of the ovum (36,37).

Of the synthetic protease inhibitors tested, N-α-tosyl-L-lysyl-chloromethane (TLCK) is the most effective (34). Fortunately, TLCK is a very poor inhibitor of plasminogen activators (38), and in fact plasminogen is often activated in the presence of TLCK or macromolecular inhibitors to prevent autolysis of plasmin (19,20,38). Thus an inhibitor similar to TLCK would not inhibit drug release by seminal plasminogen activator from the solid support, but would inhibit

fertilization. Because of the need of an arginine-valine bond in the linkage, the tripeptide Arg-Val-Lys CH_2Cl would be bound to the nylon support which when exposed to seminal plasminogen activator would result in the release of the acrosin inhibitor Val-Lys CH_2Cl.

Rationale

By taking advantage of the biochemical properties of seminal plasma and the female genital tract, one may design an in utero contraceptive device which will release a fertilization inhibiting drug (L-lysyl-chloromethyl ketone derivative) only when sperm are present in the uterus. Drug release is dependent upon cleavage of an arginine-valine bond which links the drug to a solid support (nylon). A plasminogen activator known to be active in seminal plasma will be responsible for cleavage of this bond. This contraceptive drug delivery system may be used for other agents such as spermicides [poly(ethylene glycol)p-phenyl ether derivatives].

METHODS OF PROCEDURE

Synthesis of chloromethyl ketone inhibitor (Arg-Val-Lys CH_2Cl)

After protecting the quanidine group of arginine by the formation of nitroarginine (39), and protecting the α-amino group of arginine with the t-butyloxycarbonyl group, a peptide bond with valine is

formed utilizing dicyclohexyl carbodiimide (40). Commerically available ε-amino-tosyl-L-lysyl chloromethyl ketone is reacted with the dipeptide with dicyclohexyl carbodiimide present. The protecting groups are removed using dilute HBr (40). The tosyl group on the ε-amino group of lysine may require HBr in phenol and acetic acid for removal (41).

Attachment of drug to nylon tube

The nylon tube is partially hydrolyzed using HCl followed by crosslinking to the amino groups of the drug using gluteraldehyde (42). Note that the reaction with the synthetic inhibitor, Arg-Val-Lys CH_2Cl may be performed before the ε-aminotosyl group has been removed.

In vitro and in vivo testing

Purified seminal plasminogen activator (14) is incubated with the tubes to determine if cleavage will occur. Cleavage can be detected by ninhydrin reaction with the free amino groups.

Purified acrosin (43) activity is assayed in the presence of Val-Lys CH_2Cl using N-α-benzoyl-L-arginine ethyl ester hydrolysis assay (44).

Animal studies will include removal of the contraceptive device in some animals at various times of the menstrual cycle to determine how much of the drug remains bound. If seminal plasminogen activator

activity is too high, an inhibitor to this enzyme (ε-amino caproic acid) (45) can be included in the device to attenuate this activity.

SIGNIFICANCE

In the words of Arnold Toynbee,

> "We have been God-like in our planned breeding of our domesticated plants and animals, but we have been rabbit-like in our unplanned breeding of ourselves."
>
> --National Observer, June 10, 1963.

Possibly through safe, inexpensive, easy to use contraceptive devices we may all become more God-like in our breeding behavior.

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PROPOSITION III

INTRODUCTION

Objective

Condensation products of biogenic amine metabolism, formed in the presence of acetaldehyde the primary metabolite of ethanol, have been implicated in the addiction liability of alcoholic beverages. Attempts to isolate these condensation products, which are morphine-like opiates from the brains of animals administered alcohol, have failed to date. An indirect method of determining the presence of opiate compounds in the brain is proposed to test the validity of the importance of opiates in alcoholic addiction.

Background

The neurophysiological changes associated with alcohol dependence at the cellular and molecular levels are not known. A possible biochemical foundation for this disease has been proposed based on the interactions between ethanol, its metabolite, acetaldehyde, and the neurotransmitters, norepinephrine, dopamine, and serotonin (1-6).

The deamination of biogenic amines results in the formation of their respective intermediate aldehydes through the action of monoamine oxidase. Normally, these intermediate aldehydes are further oxidized by the NAD-linked enzyme, aldehyde dehydrogenase, to the corresponding acids.

After ethanol administration to human subjects, the oxidation of the aldehyde derivative of exogenously administered [^{14}C]norepinephrine to [^{14}C]3-methoxy-4-hydroxymandelic acid was depressed while reduction of the intermediate aldehyde to [^{14}C]3-methoxy-4-hydroxyphenylglycol was enhanced (7). Ethanol ingestion evoked a similar alteration in humans of [^{14}C]serotonin metabolism resulting in decreased formation of [^{14}C]5-hydroxyindoleacetic acid and increased formation of [^{14}C]5-hydroxytryptophol (8). A comparable modification in the metabolism of endogenous epinephrine and norepinephrine in humans was reflected by a reduced urinary excretion of 3-methoxy-4-hydroxymandelic acid and an elevated excretion of 3-methoxy-4-hydroxyphenylglycol following alcohol consumption (9). The ethanol-induced interruption of oxidative catabolism and enhancement of reductive metabolism of the intermediate aldehydes derived from serotonin or catecholamines implies impaired activity of aldehyde dehydrogenase (3) via competitive inhibition of this enzyme by acetaldehyde derived from ethanol metabolism, as has been demonstrated *in vitro* (10,11) and *in vivo* (12). Acetaldehyde has been found in the brain tissue after ethanol administration in the rat (13) and the mouse (14). The acetaldehyde could be produced locally in the brain or carried to the brain from the liver.

Based on these observations, Davis and co-workers (2,3) proposed that the elevated brain concentrations of acetaldehyde following alcohol consumption would result in increased amounts of 3,4-dihydroxyphenylacetaldehyde (derived from the normal oxidative deamination of

dopamine) by saturating aldehyde dehydrogenase (which would normally oxidize 3,4-dehydroxyphenylacetaldehyde to 3,4-dihydroxyphenylacetic acid). With excess 3,4-dihydroxyphenylacetaldehyde in the brain, condensation with dopamine can occur resulting in the formation of tetrahydropapaveroline, which could undergo further biotransformation to morphine or morphine-like alkaloids. This in vivo formation of tetrahydropapaveroline is proposed by Davis to be the biochemical basis of alcohol addiction.

Independently, Cohen and Collins (1) proposed that the tetrahydroisoquinoline condensation products of acetaldehyde with biogenic amines may underlie ethanol dependence.

To test these hypotheses, an attempt was made to demonstrate the in vivo biosynthesis of tetrahydroisoquinoline alkaloids in brain tissue of rats following acute administration of ethanol (15). The animals were pretreated with pyrogallol, which was previously shown to elevate ethanol-derived acetaldehyde blood concentrations in rats tenfold higher than usual acetaldehyde blood concentrations in the absence of pyrogallol (16). Under these conditions and using gas chromatograph-mass spectrometry, salsolinol (the tetrahydroisoquinoline derivative from direct condensation of acetaldehyde with dopamine) was detected in dopamine-rich parts of the brain. Tetrahydropapaveroline was not detected nor was salsolinol detected in brains not pretreated with pyrogallol.

In another study, rats were allowed to drink one of four solutions: water, ethanol solution, levadopa solution, and ethanol plus

levadopa solution (17). The brains were analyzed for the presence of tetrahydropapaveroline by gas chromatography -mass spectrometry. In animals treated with levodopa, tetrahydropapaveroline was detected. Animals receiving levodopa and ethanol showed an enhanced formation of tetrahydropapaveroline. Neither the control (water) rats nor those receiving ethanol alone showed any evidence of tetrahydropapaveroline formation in brain tissue. The failure to detect tetrahydropapaveroline in the brain following ethanol consumption may be due to rapid metabolism of the alkaloid, by catechol-O-methyltransferase (18) or its further conversion to morphine-like alkaloids (2,3) or to the possible occurrence of the more favorable reaction of dopamine with ethanol-derived acetaldehyde to form salsolinol instead of tetrahydropapaveroline (19).

Whether the nanogram quantities of salsolinol and tetrahydropapaveroline formed in vivo in rat brain tissue under the optimized conditions described above are of any biological significance in terms of addiction potential to ethanol remains open to question.

In a recent study, tetrahydropapaveroline was delivered directly into the cerebral ventricle of rats (20). The animals were given access to both water and ethanol solution. Within three to six days of the start of the infusion of tetrahydropapaveroline, the rats, which normally rejected alcohol, drank alcohol solutions in increasingly excessive amounts. This was accompanied by symptoms that were similar to those of withdrawal and intoxication.

These results demonstrate that a condensation product of a biogenic amine induces drinking of alcohol in rats. However, that such condensation products exist in the brain after chronic drinking of alcohol, has yet to be shown.

Tetrahydroisoquinoline alkaloids and other morphine-like opiates have specific binding sites in brain tissue (21-24). The existence of specific opiate receptor sites strongly indicates the presence of natural morphine-like substances in the brain. Indeed, morphine-like peptides called enkephalins have been extracted from the brains of many vertebrate species (25-29). Enkephalin activity has a regional and subcellular distribution similar to that of the opiate receptor (30) suggesting that the enkephalins are neurotransmitters or neuromodulators associated with this receptor.

Kosterlitz and Hughes (31) proposed that a large dose of morphine would substitute for the physiological effect of enkephalin and, by a feedback message, cause the enkephalinergic neurons to reduce their activity. By analogy to what is found for other neurotransmitters (32,33), this reduction in activity should be accompanied by an increase in the levels of the transmitter. These expectations were confirmed in experiments with rats addicted to morphine (34). In the course of becoming addicted to morphine the rat brain enkephalin levels almost doubled. When abstinence was precipitated by treatment with naloxone, a potent opiate antagonist, the animals exhibited a variety of withdrawal symptoms that subsided in about an hour, at which time enkephalin levels returned to normal.

Based on these results, enkephalin concentrations in the brain can be used to monitor the presence of opiates which bind to the enkephalin (opiate) receptors. Relatively large changes in enkephalin concentration occur with the presence of minute quantities of opiate, thus making this a very sensitive method of opiate detection. If chronic alcohol consumption is accompanied by the formation of morphine-like biogenic amine condensation products, higher than normal enkephalin concentrations should be observed.

Rationale

Present theory as to the biochemical basis of alcohol dependence requires the indogenous formation of hydroisoquinoline alkaloids in the brain. Direct methods of detecting these alkaloids in brain extracts of animals administered alcohol have failed. Conditions which increase the concentration of these alkaloids in the test animals have allowed detection, but have destroyed the validity of the study. The negative result of the direct-method approach may be due to the sensitivity of the analysis.

Brain enkephalin concentrations have been shown to increase significantly when opiates are present in the brain. Thus, if alcohol dependence is accompanied by endogenous formation of hydroisoquinoline alkaloids, higher than normal enkephalin concentrations should be observed.

This method has the added advantage of detecting any opiate which may be formed in the alcoholic brain. Since it is not known which morphine-like opiate is involved (if any) in alcohol dependence, this is a distinct benefit.

METHODS OF PROCEDURE

Young chimpanzees will be used as test animals because of the similarity of withdrawal symptoms observed in man and chimps (35), and because of the relative ease of inducing alcohol dependence in these animals (36). Following the published procedure (36), one to seven-month old chimps will be given a liquid diet with 45% of the calories from ethanol, four to five times daily at standard feeding times. After six to ten weeks the animals should show withdrawal symptoms if alcohol is removed from the diet.

After establishing alcohol dependence in a group of chimpanzees, the animals will be sacrificed along with chimps whose liquid diet did not include ethanol. Enkephalin will be extracted following the procedure of Pasternak et al. (27,37). Specific opiate binding assays (27,37) will be performed on the purified enkephalin from the brains of alcohol-dependent chimps and from the brains of control animals to determine the concentration of enkephalin.

SIGNIFICANCE

It is evident that research into the role of tetrahydroisoquinolines in alcohol dependence is essential for better understanding of the biochemical basis of this disease. If these opiates are important in addiction to alcohol, prevention of their biosynthesis may offer promising leads for a rational approach to the treatment of alcoholism.

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PROPOSITION IV

INTRODUCTION

Objective

Density-inhibited cells have been shown to turnover phosphatidylethanolamine at rates equal to cells in exponential growth while the synthesis of other phospholipids is suppressed. Since the phospholipid composition of the cells remains the same, it is proposed that phosphatidylethanolamine-enriched portions of the plasma membrane are turned over at a greater rate than non-enriched portions, and that this phosphatidylethanolamine enrichment may serve as a marker as to what portion of the plasma membrane is to be turned over. The possibility that specific plasma membrane proteins are also selectively turned over during this proposed process will be investigated.

Background

A wide variety of changes in structure and function of the plasma membrane of cultured cells has been correlated with the onset of density-dependent inhibition of growth. These include a decrease in nutrient transport (1), changes in glycoprotein (2,3) and glycolipid (4) composition, and a decrease in membrane fluidity (5).

Possibly a related observation has recently been made in specific changes in lipid metabolism in density-inhibited cultures. Using pulse labels of $^{32}PO_4^{3-}$, both Cunningham (6) and Diringer and Koch (7)

have shown a relative inhibition of the turnover of phosphatidylethanolamine and phosphatidylinositol compared with other phospholipids in density-inhibited 3T3 cells and mouse embryo cells. Because of the possibility of introduction of artifacts by growing cells in low-phosphate medium as was done in these $^{32}PO_4^{3-}$-labeling experiments, Gallaher and Blough (8) used [2-^{3}H]glycerol in similar short-term pulse-chase experiments. They observed that the onset of density-dependent inhibition of growth was correlated with an inhibition of phosphatidylcholine synthesis and a constant level or increase in phosphatidylethanolamine synthesis in both BHK-21 (hamster) and 3T3 (mouse) cells. No significant differences were observed in the overall phospholipid composition of these cells, however. Therefore, the incorporation of the radioactive precursor reflects turnover rates (8).

Why should density-inhibited cells continue to synthesize specific phospholipids at rates equal to or greater than those observed during exponential growth? The plasma membrane of density-inhibited cells is known to be turned over (9,10). In some studies of plasma membrane turnover many individual membrane proteins were observed to turn over at similar rates (9,11,12), while in other studies, different protein species had dissimilar turnover rates (10,13-15). The finding of homogeneous protein turnover prompts the conclusion that degradation occurs in bulk, as would occur when endocytosis delivers a large segment of plasma membrane to the lysosome. The finding of heterogeneous protein turnover suggests that specific portions of the plasma membrane are turned over possibly for specific cellular functions such as

nutrient intake or internalization of plasma membrane receptors as has been suggested for epidermal growth factor receptors (16). In light of the continued synthesis and degradation of specific phospholipids (phosphatidylethanolamine) by density-inhibited cells while maintaining the same phospholipid composition of these cells, these portions of the plasma membrane which are turned over should be rich in phosphatidylethanolamine. Indeed, the relative increase in phosphatidylethanolamine concentration in certain portions of the membrane may serve as a marker for that portion of the membrane which is to be endocytosed and degraded.

Rationale

Density-inhibited cells have been shown to turn over phosphatidylethanolamine at rates equal to cells in exponential growth while the synthesis and degradation of other phospholipids is suppressed. Since cells continue to turn over their plasma membrane when density inhibited and since the phospholipid composition of the plasma membrane remains the same whether the cells are density inhibited or growing exponentially, those portions of the membrane which are endocytosed and degraded should be rich in phosphatidylethanolamine. This hypothesis will be tested by autoradiography of cells whose plasma membranes contain ^{32}P-labeled phosphatidylethanolamine. The labeled phospholipid will be introduced into the membrane by phospholipid exchange proteins.

The proposed phosphatidylethanolamine enrichment of endocytic vesicles may serve as a marker to the cell as to which portions of the membrane are to be turned over. If indeed specific portions of the membrane are observed to be turned over, this may be for some specific purpose such as internalization of a specific protein. To test this hypothesis, the spectrum of iodinatible proteins in the plasma membrane will be compared with the iodinatible proteins in the purified vesicles.

METHODS OF PROCEDURE

In recent years a number of phospholipid-exchange proteins have been purified from a variety of biological tissues (17,18). These proteins exchange phospholipid between two structures without altering the phospholipid content of each structure. Thus they provide an ideal nonperturbing method of introducing a radiolabel into a membrane.

The phosphatidylethanolamine-exchange protein has not yet been purified to homogeneity. However, a partial purification of this particular exchange protein has been published (18). By incubating confluent (density-inhibited) 3T3 cells with vesicles of labeled phosphatidylethanolamine and the exchange protein, the plasma membranes of the 3T3 cells will incorporate labeled phosphatidylethanolamine without altering the relative concentration of this phospholipid.

This incubation will be followed by EM autoradiography to determine if the endocytic vesicles are enriched in phosphatidylethanolamine

as compared with the plasma membrane and if phosphatidylethanolamine patching occurs on the plasma membrane.

To insure that phosphatidylethanolamine vesicles are not endocytosed directly during the incubation of the 3T3 cells with these vesicles, a control will be done of incubating 3T3 cells with the labeled phosphatidylethanolamine vesicles but without the phospholipid-exchange protein followed by EM autoradiography.

To investigate the possibility that specific portions of the plasma membrane are internalized because of the existence of particular proteins in these portions of the membrane, the 3T3 plasma membrane proteins will be iodinated using lactoperoxidase and iodine-125 (11). The cells will then be incubated in medium containing silver iodide (19) or colloidal iron (20) to change the density of the endocytic vesicles and allow purification by ultracentrifugation in sucrose gradients (19,20).

The iodinated proteins in the purified vesicles will be separated by polyacrylamide gel electrophoresis and compared with the spectrum of iodinated plasma membrane proteins observed in whole-cell homogenates. If indeed an enrichment of a particular iodinated protein is observed in the endocytic vesicles, the hypothesis that endocytosis in density-inhibited cells only involves specific portions of the plasma membrane will be verified and a possible reason for this observation will be implicated.

SIGNIFICANCE

The mechanism of growth control in cultured cells has been a highly studied process within recent years, but remains largely not understood. A wide variety of changes have been observed to occur in cells as they approach density-inhibited growth. The interrelationship of these observations has not been elucidated. Until a more complete understanding of growth control is achieved, it is important to obtain as much information about each of these observations so that its relative importance can be evaluated.

The continued high level synthesis of phosphatidylethanolamine by density-inhibited cells is among these observations. The distinct possibility exists that this continued synthesis is important in maintenance of density inhibition, possible, as proposed here, by serving as a marker for endocytosis of the plasma membrane and any receptors which may be included in that portion of the membrane. This possibility warrants further investigation.

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PROPOSITION V

INTRODUCTION

Objective

Short chain aliphatic acids of vaginal origin have been shown to be important in primate sexual behavior. These same acids are produced in the human vagina. The relationship between specific aliphatic acid anosmia and aberrant human sexual behavior is sought in an attempt to learn the importance of these chemicals in human behavior.

Background

Pheromones are chemicals, either odor or taste substances, that are released by organisms into the environment, where they serve as messages to others of the same species (1). Pheromones are widely used by animals ranging from protozoans to primates as a primary means of transmitting information. This chemical communication system may be used for attracting a mating partner, for directing others to food, for causing others to stay away, or for other behavioral functions.

Although interest in mammalian pheromones is relatively recent, some examples of chemically induced behavior in mammals are found in the literature. A pheromone from the boar (5α-androst-16-en-3-one) elicits an immobilization reflex in oestrus sows so that they stand rigid while being mated (2,3). Dogs are attracted over distances to oestrus bitches (4) and also to their urine (5). Similarly, the

urine of oestrus mares sexually stimulates the stallion (6). Rams can differentiate between oestrus and non-oestrus ewes by their scent (7), and the male rat prefers the odor of receptive as opposed to non-receptive females (8). Thus, the evidence is gradually accumulating that olfactory communication plays an important role in mammalian reproductive behavior.

More important, as to their implications to human behavior, are animal studies with primates. Among all primate sexual attractants, the only one whose behavioral function, hormonal control, and chemical nature has been analyzed in detail is the sex pheromone of the rhesus monkey. Male rhesus monkeys were trained to press a level in order to gain access to a female partner (9). The males frequently worked to obtain access to ovariectomized females who were rendered receptive by injections of estrogen, but did not consistently work to obtain access to ovariectomized, nontreated females. When the males were rendered anosmic by plugging the nasal olfactory area, they were no longer able to discriminate between receptive and nonreceptive females. Reversal of anosmia restored their ability to do so, clearly indicating that the female's attractiveness to the male was dependent on olfactory cues.

The source of these olfactory cues was shown to be vaginal secretions (10,11) whose production is under estrogenic control (12). By means of gas chromatography and mass spectrometry the active constituents of the vaginal secretions was found to be a mixture of five aliphatic acids: acetic acid, propionic acid, iso-butyric acid,

n-butyric acid, and iso-valeric acid (13). A synthetic mixture of these acids in the same concentration ratio found in the vagina, produced the behavioral effects of the natural pheromone. These aliphatic acids have been shown to be present in the vaginal secretions of a wide range of primate species including anubis baboon, patos monkey, pigtail monkey, crabeating monkey and squirrel monkey (14).

The fact that sex pheromones act as powerful stimulants of precopulatory behavior in some higher primate species begs the question of the importance of pheromones in human sexual behavior. Humans have organs which could be part of a pheromone system (15). These include apocrine glands which are sometimes associated with tufts of hair. Some of the glands do not produce sweat and presumably have some other function. For example, the prepuce in the male and labia in the female have glands that secrete odorous materials which might be suspected as having pheromone activity.

Women are particularly sensitive to compounds having the odor of musk (16). Only sexually mature women clearly perceive musk-smelling compounds, and their sensitivity to these compounds is greatest at about the time of ovulation (17,18). Males become more sensitive to these compounds following an injection of estrogen. The compound responsible for the musk-like odor of human male urine is 5α-androst-16-en-3-one, the known boar pheromone (19). These observations provide strong circumstantial evidence for the existence of a male sex pheromone. One possible effect of a male sex pheromone is the often observed menstrual synchrony of women living together in a college

dormitory (20).

Evidence is also accumulating to support the likelihood that human females produce sex pheromones. Two recent studies have shown a differential response by infants to the odor of their mother and that of a stranger (21,22). In addition, the volatile fatty acids of primate vaginal origin which have been shown to operate as sex pheromones in rhesus monkeys, are also produced in the human vagina (23-25). The concentration of these acids varies during the menstrual cycle, reaching a peak near the time of ovulation. Women who take oral contraceptives produce lower amounts of the acids and do not exhibit the rhythmic concentration change during the menstrual cycle.

If these aliphatic acids are important in human sexual behavior, aberrant sexual behavior would be expected in those individuals unable to perceive them. Specific anosmia for short chain aliphatic acids is known to exist in man (26). Specific anosmia is defined as the condition in which a person of otherwise normal olfactory acuity cannot perceive a particular compound at a concentration such that it is obvious to most other people (27). The aliphatic acid anosmics should prove very useful in the study of the effects of volatile vaginal aliphatic acids on human sexual behavior.

The possible aberrant behavior of anosmic individuals could take many forms. From the primate studies, total lack of male interest in females resulted when animals were artificially rendered anosmic (9). Certainly a similar study conducted on human males would have a

different result. However, true human aliphatic acid anosmics have never experienced vaginal pheromonal input. If pheromonal cues are important in sexual object-choice as has been found for other mammals (28,29), lack of sensory input from birth may have a dramatic effect on the sexual behavior of the individual. This aberrant behavior might be expressed as homosexuality, impotence, or more subtle abnormal behavior.

Rationale

Several compounds have been implicated as having pheromonal activity in humans. Among these, the aliphatic acids of vaginal origin have been characterized to the greatest extent. Approximately 3% of humans are anosmic for short-chain aliphatic acids (26). If these volatile acids are important in human sexual behavior, a positive correlation is expected between aliphatic acid anosmia and aberrant sexual behavior. How this aberrant behavior would be manifested is not clear. As an initial study, the relationship between short-chain aliphatic acid anosmia and homosexuality is proposed.

METHODS OF PROCEDURE

A large adult male population will be necessary to obtain reasonable statistics for this study. Fortunately, the screening for aliphatic acid anosmia is easily performed. Following the technique

of Amoore (30), each subject is given a set of five stoppered Erlenmyer flasks. Two of the flasks contain isovaleric acid in solution, and the other three contain pure water. If the subject correctly identifies both odorous flasks, he is tested again with a lower concentration. If he detects no odor in the first test, or makes a wrong selection, he is tested with a higher concentration. The lowest concentration step correctly chosen defines his olfactory threshold.

Each participant will be asked to fill out a questionnaire which will estiablish if he is an overt homosexual or if other aberrant behavior exists (impotence).

SIGNIFICANCE

The notion of pheromonal influences on man is speculative, but if it is true, it could be important for medicine and psychiatry. In the proposed study, a positive correlation between aliphatic acid anosmia and aberrant sexual behavior would have far reaching implications. First, a functioning pheromonal communication system would be shown to exist in man. Second, the aliphatic acid pheromone of vaginal origin would be shown to be important in sexual preference. Third, the biochemical basis for one aspect of human behavior will be ascertained. The potential for increased understanding of human behavior certainly justifies research in human pheromonal communication.

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