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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

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Inhibition in vitro and Plasmid DNA 3'-Terminal Analysis

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF: Bachelor of Science in Biochemistry

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INHIBITION IN VITRO
Plasminogen N-Terminal Analysis

by
Wayne E. Henderson

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MATERIALS AND METHODS

Materials - The synthesis, purification and characterization of PDZ has been described (27). Stock solutions of 0.2-1mM were prepared in redistilled dimethylformamide (DMF). Further dilutions were made into PBS before each experiment. PDZ was used in the same manner. Plasminogen was purified from dog plasma as previously described (28). Trasylol was purchased from Mobay Chemical Corporation. PBS contained 0.137M NaCl, 2.68mM KCl, 8.00mM Na₂HPO₄, 1.47mM KH₂PO₄, 0.91mM CaCl₂, and 0.49mM MgCl₂ at pH 7.2.

p-Nitrophenyl-p-guanidinobenzoate (NPGB) was purchased from ICN and dissolved in redistilled DMF to various concentrations. Further dilutions were made in PBS before each experiment. All electrophoresis reagents were purchased from Biorad. The discontinuous pH, SDS-polyacrylamide gel system described by Lasenby was used (29). Densitometer tracings were obtained using an Ortec densitometer. Diisopropyl fluorophosphate (DFP) was purchased from Aldrich. Lysozyme, bovine serum albumin (BSA), pyroglutamate aminopeptidase, dansyl chloride, and the dansylated amino acid standards were purchased from Sigma. Dansyl-amide, dansyl-cysteic acid and Cheng Chin polyamide sheets were purchased from Pierce. The dansylated standards were dissolved in 3:2 acetone:acetic acid to about 1 mM, their TLC behaviours determined, and then mixed in equal volumes. Bovine pancreas trypsin, three times crystallized, was purchased from Worthington. Stock solutions were prepared in 1mM HCl and stored at
-20°. An active site titration with NPGB indicated 74% of the total protein (determined by absorbance) was active trypsin.

**Cell Culture** - The R74-71-D2 cell line was a kind gift from Dr. N. Sueoka of the University of Colorado, Boulder (30, 31). The cells were cultured in 100 x 15 mm flasks (Lux) in a Forma Scientific incubator set at 37° and 5% CO₂ atmosphere. The culture medium used was Dulbecco's Modified Eagles' Medium (Gibco) with 2.5% fetal calf serum (Gibco), 12.5% horse serum (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were passaged twice weekly at semi-confluency using trypsin-EDTA in Hank's balanced salt solution (Gibco). The number of cells in a suspension was determined with a Coulter counter.

**PA Assay** - At least sixteen hours prior to performing an assay, 0.10 ml aliquots of a cell suspension in complete medium were placed into microwells (Costar 6.4 mm diameter tissue culture cluster dishes or Kimble 9 mm O.D. shell vials). After this seeding period, the medium was removed and the cells treated as described herein. The cells were then washed three times with PBS containing 4 mg/ml BSA. To each microwell, 50μl of PBS containing plasminogen - usually at 0.5 mg/ml - were added. After incubation at 37° for 30 minutes, the 50μl aliquot was withdrawn and added to 50μl of PBS containing 2.66μM PDE. After 10 minutes at room temperature, the sample was diluted to 1 ml with PBS and the magnitude of the fluorescent burst was measured using a Perkin-Elmer MPF-44A fluorescence spectrophotometer with a Universal Digital Readout or a Perkin-Elmer 650-40 fluorometer. The excitation and emission wavelengths were 491 nm and 514 nm, respectively, both
set with a band width of 4 nm. The change in fluorescence ($\Delta F$) is equal to the magnitude of the fluorescence in the sample cuvette minus the magnitude of the fluorescence in a reference cuvette containing the same concentrations of FDE and plasminogen but in which the plasminogen had been incubated in microwells without cells. $\Delta F$ is proportional to the plasmin concentration.

**Amino Terminal Analysis** - The procedure used for amino terminal analysis was as described by Gros and Labouesse (32) with minor modifications. The TLC detection method was similar to that of Woods and Wang (33). A method of reduction and carboxymethylation was adapted from Stitt's procedure (34) and a procedure to remove pyroglutamic acid from the amino terminus of a protein was as described by Podell and Abraham (35).

Plasminogen, initially in PBS at approximately 10 $\mu$g, was diluted to 1 $\mu$g with water and 3 ml of this dialyzed at 4$^\circ$ first against water and then against the reduction and carboxymethylation buffer (RCMB: 1.44M tris-HCl, 5M urea, 1mM EDTA, pH 8.6). To this, 75ul of 0.36M dithiothreitol (DTT) in RCMB were added and the tube was tightly capped after flooding it with nitrogen. After 4 hr at room temperature, 300ul 0.36M iodoacetate in RCMB were added for a 20 minute incubation at room temperature in the dark. Addition of 23.9ul of $\beta$-mercaptoethanol quenched the reaction.

The reduced and alkylated protein was dialyzed at 4$^\circ$ against deblocking buffer (DBB: 0.1M phosphate, 5mM DTT, 10mM Na$_2$EDTA, 5% glycerin v/v, pH 8.0) and split into two 1.3 ml portions. To one portion was added 125ul of 4 $\mu$g pyroglutamate aminopeptidase in DBB.
The other portion received identical treatment throughout the procedure but was not exposed to the enzyme. The tubes were flooded with nitrogen, sealed and incubated at 4°C for 9 hr. After equilibration to room temperature, 125 ul more of the enzyme solution were added to the previously exposed portion prior to a 14 hr. room temperature incubation. Dialysis against water at 4°C and concentration to 1.5 M prepared the samples for dansylation.

The dansylation procedure began with 300 ul of the reduced, alkylated and 'deblocked' plasminogen or with 50 ul of approximately 9 M plasminogen in PBS diluted to 300 ul with water. In either case, the sample was added to 250 mg of Mann Ultrapure urea, 150 ul of 0.4M phosphate at pH 8.5 and 250 ul of redistilled DMF. The protein was dansylated by adding 100 ul of 10 M dansyl chloride in dried acetone for a 2 hr incubation under darkness at room temperature. After adding 10 ml of cold 10% TCA, the sample was centrifuged at 5000 rpm for 15 minutes. The TCA was poured off and the pellet washed once with 1M HCl and once with acetone. Resuspension of the precipitate in 500 ul of 6M HCl and transfer to a hydrolysis vial was difficult because of the stickiness of the product. The vial was sealed and incubated at 110°C for 4 hr. The material was then taken to dryness in a vacuum concentrator prior to resuspension in 3:2 acetone:acetic acid or in a 0.1M ammonium formate buffer at pH 3.5. An ether extraction from the latter solvent leaves the non-specific products - dansyl-OM, ε-dansyl lysine, ε-dansyl tyrosine and other, non-fluorescent products - behind in the aqueous phase. The ether was then evaporated and the precipitate dissolved in 25 ul of 3:2 acetone:
acetic acid.

A few microliters of the sample were spotted onto a 5 x 5 cm polyamide sheet and the first dimension (200:3 H₂O:formic acid) run with the origin at the lower right corner. The second dimension (9:1:1 benzene:pyridine:acetic acid) was run with the origin at the lower left corner. Complete drying between steps is crucial to prevent tailing and smearing of the spots. The volume spotted was varied to maximize the intensity of the spots and yet avoid overloading the TLC sheet.
The original observation of enhanced proteolytic activity of tumors was made in 1925 by Fischer (1). He observed that chicken sarcomas growing in vitro could lyse fibrin clots whereas normal cell explants could not. Recently (2-5), this fibrinolytic activity of tumors was shown to be a consequence of a serum factor (plasminogen) and a cell factor (plasminogen activator or PA). Thus, in Fischer’s experiments, the PA of the sarcoma cells cleaved plasminogen in the serum to plasmin which then lysed the fibrin clots. Normal chicken cells exhibited little or no PA activity. Since these observations, increased levels of PA activity have been found to be characteristic not only of malignant cells growing in vitro (2, 6-9) but also of normal cells involved in tissue remodeling and migration (10). Furthermore, PA synthesis has been shown to be inducible by tumor promoters (11, 12) and carcinogens and to be modulated by the presence of physiological concentrations of hormones (14-18).

The nature of the correlation between PA activity and tumorigenicity is unknown: What functions does PA perform for the
transformed cell? Is PA activity necessary and/or sufficient to initiate and/or maintain the transformed state? Is the only purpose of PA to activate plasminogen to plasmin? What is the normal, physiological role of the enzyme?

Cleverly used tools are needed to extract answers to all of these questions. Classically, one of the best tools for determining protein function has been genetic manipulation. The genetics of eukaryotes are not sufficiently understood to allow this simple approach and, although bacterial activation of plasminogen has been observed (unpublished observation), any meaningful model system must employ eukaryotic cells.

The tool of choice, then, is a method of inhibiting the enzyme. If the enzyme is necessary for cellular growth, then an inhibitor provides a selective pressure allowing only overproducers of the enzyme to survive. If not required for cell proliferation, then inhibition of the enzyme followed by the screening of high level producers from the general population provides a means to the same end; comparison of cells in which the primary difference is the activity of the enzyme.

Ideally, an inhibitor should bind quickly, tightly and reversibly to the enzyme under physiological conditions and at concentrations low enough to be selective for only the enzyme and not toxic by some other means. If the inhibitor is stable to physiological conditions, it would be an easy matter to maintain a constantly inhibited level of the enzyme by merely introducing the inhibitor into the culture medium.

The serine protease inhibitor NPGS (19, 20) seemed likely to meet
some of these criteria. In the presence of NPGB, a serine protease is rapidly inactivated by acylation and then slowly deacylated. Furthermore, NPGB is relatively stable in aqueous buffers and has been reported to be the most sensitive known inhibitor of PA (21).

Thus, an attempt was made to continually inhibit cellular PA by merely incorporating NPGB into the culture medium. Of course, the first question was whether NPGB is stable in complete medium under incubator conditions. The answer is no; Fig. 1. The half-life of NPGB is in fact less than 20 min under these conditions. This simple approach was thus ruled out unless, once acylated, the PA of cells deacylates slowly enough as to remain inhibited between occasional treatments with NPGB.

To determine the first order rate constant, $k_3$, for deacylation is not a simple matter because PA can not be instantaneously assayed. The amount of active PA present after a treatment with NPGB is steadily increasing and will not be the same at the end of an assay as it was at the beginning. A general method for eliciting a $k_3$ from indirect, fixed-time assays was developed.

After a treatment with NPGB, the amount of free PA at any time is

$$E(t) = E(1 - e^{-k_3(t+t_0)})$$

where $E_0$ is the level of activity of untreated cells, $t$ is the time following removal of NPGB and $t_0$ is the time separating the removal of NPGB and the first appearance of active PA. (For instance, if PA activity is 50% of normal when NPGB is removed ($t=0$), then $t_0 = t_{1/2}$. )
The rate of formation of the second enzyme, plasmin, is directly proportional to $E(t)$ by the constant $\frac{dP}{dt}$ so that

$$\frac{dP}{dt} \bigg|_{t=\infty} = (1-e^{-k_3(t_0+t)}) \quad (1)$$

See Fig. 2A. Integration of this expression over the assay interval $t_1$ to $t_2$ gives the amount of plasmin (or fluorescent units) formed during that interval; the quantity actually measured in the assay.

$$P_2 - P_1 = \Delta FU = \frac{dP}{dt} \bigg|_{t=\infty} \left[ \Delta t_{1,2} + \frac{1}{k_3} (e^{-k_3(t_2+t_0)} - e^{-k_3(t_1+t_0)}) \right] \quad (2)$$

See Fig. 2B. The constant $\frac{dP}{dt} \bigg|_{t=\infty}$ is determined by performing the assay on untreated cells for a convenient interval of time, $\Delta t_\infty$. Then

$$\Delta t_{1,2} = \Delta FU + \Delta t_\infty = -\frac{1}{k_3} (e^{-k_3(t_2+t_0)} - e^{-k_3(t_1+t_0)})$$

For the results presented here, both $\Delta t_{1,2}$ and $\Delta t_\infty$ are 30 min so

$$50(1-\Delta FU) = -\frac{1}{k_3} (e^{-k_3(t_2+t_0)} - e^{-k_3(t_1+t_0)})$$

A computer program was written to iteratively solve this equation for $k_3$ for each of nine data points, to then determine the mean and standard deviation of the nine values and then to increment $t_0$ and repeat the procedure. The mean and standard deviation are plotted vs. $t_0$ in Figures 2C and 2D respectively. Taking the mean value where the
standard deviation is at a minimum \( t_0 = -7 \) min gives
\[
k_3 = (1.9 \pm 0.2) \times 10^{-2} \text{ min}^{-1}
\]
indicating that the half-life of the acyl-enzyme complex is about 17 min in 4 \( \text{mg} \) BSA in PBS. An experiment in which deacylation was allowed to occur under complete medium showed that essentially complete activity was present following 30 min under medium and a 30 min assay with plasminogen. This indicates that deacylation is even faster in medium.

A \( t_0 \) of -7 min is a curious result in that it implies no PA activity appears for seven minutes after removal of NPGB. This, and the possibly related observation that any assay begun directly after the removal of NPGB showed a signal below background, prompted the thought that perhaps NPGB was sticking to the microwell surface and later leaching off to inhibit plasmin.

To investigate this hypothesis, an empty microwell was treated for 10 min with 50\( \mu \)M NPGB and washed three times with PBS. A 100\( \mu \)l aliquot of 0.1\( \text{mg} \) BSA in PBS was immediately added to the microwell and allowed to stand for 20 min. This aliquot was then withdrawn and added to 50\( \mu \)l of a plasminogen solution which had been exposed to cells and thus contained plasmin. For a control, 100\( \mu \)l of the BSA solution were added to 50\( \mu \)l of the solution containing plasmin. After 10 min at room temperature, the mixture was titrated with FDE prior to dilution to 1 ml with PBS. The control gave a \( \Delta \text{FU} \) of 279 while the sample exposed to the NPGB treated microwell gave a signal below the plasminogen background (which was obtained from a sample similar to the control but with plasminogen not exposed to cells).

So long as the washing procedure is not grossly inefficient, then
the sticking of NPGB to the microwell is strongly implicated. To distinguish inefficient washing from sticking, empty microwells were treated with 50μM NPGB for 10 min and then washed repeatedly with 230μl aliquots of 4% BSA in PBS using an Eppendorf pipette. Each wash was added to 100μl of a trypsin solution and allowed to incubate 10 min before titration with 50μl of an FDE solution. A control using 230μl of the stock BSA solution gave a ΔFU of 920. An aliquot of the BSA solution which stood in an untreated microwell for 15 min caused no inhibition of the trypsin signal. The first wash caused total inhibition of the trypsin signal and the signal gradually increased with each successive wash. The sixth wash gave a ΔFU of 860. The seventh wash was allowed to stand for 10 min in the microwell and gave a FU of only 390. The fact that more NPGB appeared in the seventh, longer wash than did in the sixth wash shows that NPGB must be sticking to the microwell and later leaching off.

How much later? The curve in Figure 4 shows that the majority of the NPGB leaves the microwell within 7 min and then continues to slowly leach off into a fourth wash.

Some attempts to characterise and circumvent the problem of NPGB sticking to microwells are summarised in Table 1. The procedure for these experiments began with a pretreatment of the microwell with 200μl of a test solution for 5-10 min. A microwell was then treated for 5 min with 100μl of a 2% DMF, 200μM NPGB solution in PBS which also contained a material being tested for its ability to prevent the sticking phenomenon. After removal of the treatment solution, the microwell was washed once with 200μl of a test solution and then twice
more with PBS before 50μl of a trypsin solution were added for 5 min. The trypsin was then removed, titrated with FDE, diluted to 1ml with PBS and the fluorescence measured. Standard conditions are thus no pretreatment followed by treatment with only the DMF, NPGB solution and three consecutive PBS washes. The control microwell for any experiment was treated exactly as the test microwell was but with a treatment solution lacking NPGB.

In addition to these experiments, polylysine treated (22) microwells and Superbeads (23), both of which reportedly provide a positively charged surface for cell attachment in contrast to the negative charge of a microwell, were tested for their ability to resist NPGB sticking. Neither of these surfaces appeared to offer any significant advantage over the untreated microwells.

From the information in Table 1, it seemed as if the pH of the wash was the only factor which showed a decisive effect on the problem. Including a first wash of pH 2 PBS as part of a solution to the problem seemed reasonable for two reasons: cells treated with pH 2 PBS showed no immediately adverse reactions and the acyl-enzyme complex is reportedly stabilised by acid pH as opposed to being destabilised by alkaline pH (24). Preliminary experiments had shown that NPGB does not stick as tenaciously to glass as to the polystyrene microwells and so it was thought that a combination of using glass vials to grow the cells in and a first wash with pH 2 PBS after an NPGB treatment might prevent the later appearance of NPGB in the fluid above the cells.

Empty glass vials were treated with NPGB and washed once with pH
2 PBS and twice with PBS. Trypsin added to this vial showed no inhibition had occurred when the aliquot was later titrated with FDE. Was the problem solved?

A glass vial containing cells was treated with 50uM NPGB for 20 min, washed twice (by accident) with pH 2 PBS and twice more with pH 7.2 PBS before 50u1 of trypsin were added for 10 min. A similarly treated microwell not treated with NPGB showed a FU of 610 in the trypsin aliquot after titration with FDE. The trypsin aliquot from the treated microwell gave no signal. Not only did the problem return with the addition of cells but it apparently increased in magnitude. It seems that NPGB sticks to the cells themselves.

In order for the $k_3$ presented earlier to be accepted as a valid approximation of the true value, the ability of NPGB to inhibit PA had to be verified. If PA is inhibited by NPGB, then the later appearance of activity as observed is almost certainly due to deacetylation. If the ability of NPGB to inhibit PA is questionable, then an alternate explanation of the data is a constant, uninhibited level of PA producing a certain amount of plasmin which is then inhibited to lesser degrees by NPGB leaching from the microwell wall.

An attempt to differentiate these alternatives involved allowing FMK and NPGB to compete for any plasmin that might be generated during an assay with plasminogen containing both of the titrants. The appearance of a fluorescent signal would indicate plasminogen activation and thus incomplete inhibition of PA. Although the stability of NPGB in a plasminogen solution and the quantitative relationship of the FMK to NPGB ratio with attenuation of the plasmin signal are unknown, the results presented in Figure 3 indicate that plasmin was
generated under the conditions of the experiment. The ability of NPGB to inhibit activator fell further into doubt.

As an aside, it was questioned whether FME also sticks to polystyrene. Microwells treated with an FME solution were washed three times with PBS and once with base. The basic wash was diluted to 1ml with PBS and the fluorescence measured. Approximately 5% of the potential fluorescence appeared in the basic wash indicating that FME does indeed stick to microwells.

To further investigate the ability of NPGB to inhibit PA, an SDS PAGE was performed on plasminogen samples which were incubated over cells in the presence of Trasylol and varying concentrations of NPGB. See Figure 6. (A preliminary gel showed that Trasylol does not inhibit PA at the concentration used. This was also verified by an experiment in which cells treated with a solution of 5% Trasylol in PBS and assayed immediately afterwards showed full PA activity.) The results of this experiment explain why inhibition of PA was not observed in the previous experiment; a concentration of 1μM NPGB has little effect on PA activity. The results also show, assuming the inhibition is fast, that the treatment used to inhibit PA in the k₃ experiments was more than sufficient to inhibit most of the PA activity. Pretreating cells with NPGB before adding the plasminogen containing NPGB would test the assumption. If the inhibition is not fast, there will be less plasmin formed by the pretreated cells as compared to cells not pretreated with NPGB. If the assumption is valid, then the appearance of significant activity within an hour of NPGB removal necessitates a deacylation rate on the order of the
one reported.

Returning to the problem of maintaining an inhibited level of PA activity, it can be seen that the use of NPGB would present difficulties. The inhibitor is not stable in medium nor is the acyl-enzyme intermediate it forms. Frequent treatments with fresh NPGB would be necessary to maintain a low level of PA activity. With the possible availability of other inhibitors to accomplish this, the effort and expense introduced by NPGB did not seem worthwhile.

Rather, interest turned toward the possibility of using DFP to inhibit PA. One appeal of DFP is that the inhibition should be essentially irreversible; allowing very infrequent treatments. DFP is well known to inhibit serine proteases.

Does DFP stick to a microwell surface? Dry microwells were treated with 75mM DFP in PBS or with PBS only for 5 min, washed three times with PBS and assayed for remaining DFP by adding 50ul of a trypsin solution for 5 min. After titration with FDE, the untreated and the DFP treated microwells both gave a ΔFU of 450. A similar experiment with 10mM DFP for 1 hr and a trypsin incubation of 20 min gave the identical conclusion that assays for PA should not be affected by DFP interacting with the microwell surface.

The next questions were, as with NPGB, regarding the stability of the inhibitor and its inhibited enzyme product. Five rows of three microwells each were treated as follows: All were washed three times with 4% BSA in PBS and PBS was added to rows 1-3 while 80mM DFP in PBS was added to rows 4 & 5. After 30 min in the incubator, PBS was withdrawn from rows 2 & 3 and fresh DFP was added to row 2. The used
DFP from row 5 was withdrawn and added to row 3. Row 1 remained under PBS, row 4 under DFP and to row 5 was added PBS. After 30 more minutes in the incubator, all the microwells were washed three times with the BSA solution and assayed for PA by a 60 min incubation with plasminogen. Table 2 shows the results. As row three indicates, only 31% of the PA activity was inhibited by the used DFP as opposed to 67% inhibition by the fresh DFP. Rows 2 & 4 confirm this observation. The effective concentration of DFP must drop during such a treatment.

A comparison of rows 2 & 5 shows little or no return of PA activity in 30 min under PBS. This implies that the $k_3$ reported earlier is in fact for deacylation and not the return of activity by some other means.

To further characterize the return of PA activity following a DFP treatment, an experiment was performed as follows: Either 50 or 75ul of 80mM DFP in PBS was added to microwells containing D2 cells from which the medium had been removed. After thirty minutes, 150ul more of the DFP solution were added. In thirty more minutes in the incubator, the used DFP was withdrawn and the procedure repeated. Once the second 150ul of DFP had been added, an hour elapsed before the cells were washed three times with 4 mg/ml BSA in PBS and either assayed or placed under medium. Those cells assayed immediately showed no PA activity.

Under the microscope, treated cells soon appeared different than their untreated counterparts and began to detach from the microwell. An attempt to count the cells showed an abnormal size
distribution profile with the preponderance of counts in the smaller size range. From these and other observations it was concluded that high concentrations of DFP are toxic to D7 cells. Even a 15 min treatment with 57mM DFP in PBS, which inhibited 47% of the PA activity, showed some ill effects.

Attempts to lessen the toxicity included mixing the DFP into complete medium and adjusting the pH of the DFP solution. Neither attempt showed a distinct advantage; the combination of DFP in medium actually seemed more toxic. Cells treated for 15 min with 50µM NPGB were no better suited to withstand a later treatment with 57mM DFP for 15 min than were untreated cells. This implied a toxicity unrelated to PA inhibition and suggested lowering the concentration of DFP.

Medium was removed from microwells containing cells and 200µl of 10mM DFP in PBS added for a 1 hr treatment in the incubator. The cells were then washed three times with 4 mg ml⁻¹ BSA in PBS prior to being assayed or being placed under medium. Control cells were under PBS for 1 hr and similarly treated. The treated cells showed 79% inhibition when assayed (in duplicate) immediately and 71% when assayed after being in medium for 1 hr. The treated cells appeared normal at the time of the experiment and 24 hr later. A treatment as used before in which the DFP is refreshed occasionally over a 2 hr period would be expected to give more complete inhibition and may be useful for inhibiting PA and yet maintaining viable cells.
Amino terminal analyses of dog and human fractions I and II were performed on the native protein and on the reduced, carboxymethylated and pyroglutamate aminopeptidase treated plasminogen. The TLC system used for identification of the dansylated amino acids clearly separates the standards used except for proline and valine which comigrate. See figure 7. Since a dansyl-glutamic acid spot was clearly visible in the sample not exposed to the deblocking enzyme, and because no significant differences were observed between the native protein and the treated samples, the lengthy procedure of deblocking does not seem justified. Figure 7 shows approximate sketches of the TLC plates and the general features observed. Any single plate generally had a number of unidentified, faint spots. On the basis of these plates, it seemed reasonable to conclude that both fractions I and II contain some mixture of Glu and Lys plasminogen. The plasminogen purified from dog blood appears to have a higher ratio of Lys to Glu plasminogen than does the plasminogen of human origin. (A previous report (25) claims the amino terminus of dog plasminogen is blocked.) If the intensity of a TLC spot is relative to the amount of either species present then human plasminogen is predominately Glu plasminogen but with a detectable proportion of Lys plasminogen. The amino terminal analysis of lysozyme, which is known to have a lysine N-terminus (26), showed a clear di-dansyl-lysine spot in addition to the non-specific spots.
The availability of a method to inhibit the plasminogen activator of living systems is a crucial advance towards determining the functions of this enzyme. Experiments presented here have shown that NPCB and Di may both be used to inhibit the PA of living cells. The use of either, however, involves certain difficulties.

NPCB, for instance, is unstable in culture medium as is the acyl-enzyme intermediate it forms upon interaction with PA. To maintain an inhibited level of PA activity with NPCB would require frequent treatments with the inhibitor. Furthermore, the difficulty in using NPCB as a PA inhibitor is accentuated by its ability to stick to - and later leach off of - the culture surface and even the cell surface. DFP, an analogue of NPCB, also sticks to a microwell surface.

Despite these hindrances, NPCB can be used to inhibit the PA of live cells and has some potentially useful properties. A relatively low concentration of NPCB will inhibit PA and the inhibition is reversible.

DFP, on the other hand, appears to give a nearly irreversible inhibition. The major difficulties in using DFP are its toxicity to cells at higher concentrations and the lengthy treatment required to inhibit PA. During such a treatment, the effective concentration of DFP declines. DFP does not appear to stick to the microwell surface.

An amino terminal analysis was performed on various plasminogen samples. Reduction, carboxymethylation and 'debloking' were performed and later deemed unnecessary. Both fraction I and fraction II plasminogen, whether of human or dog origin, are made up of a population of Glu and Lys species. Dog plasminogen seems to have a higher ratio of Lys to Glu than does human plasminogen. Plasminogen from either source appears to be predominately Glu.
Bibliography

- 20 -


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FIGURE LEGENDS

Figure 1 The decay of NPG in complete medium under incubator conditions is first order. A 150uM solution of NPG in DMF was diluted to 150uM with complete medium at t=0 and the A_{400} recorded at the indicated times. For each reading, 500ul of the reaction were diluted with 1500ul of a pH 8.3, 20mM CaCl_2, 0.1 sodium barbital buffer and the A_{400} measured vs. an identical blank lacking only NPG. This blank had an A_{400} of 0.12 vs. H_2O. The % NPG remaining is defined as 100(A_t/A_0) ; A_0 is the average of three readings at t=135 min where greater than 99% of the NPG has decayed. The decay shows a half-life of 19.1 minutes.

Figure 2A The rate of plasmin formation increases as PA deacylates following complete inhibition at t=0. A k_3 of 1.9x10^{-2}min^{-1} and a t_0 of zero gives the curve shown. See equation no. 1

Figure 2B Complete acylation of PA at t=0 followed by deacylation with a k_3 of 1.9x10^{-2}min^{-1} causes the total amount of plasmin which could have been formed since t=0 to follow the shown curve. The rate of fluorescent unit formation (plasmin formation) expressed by inhibited cells, dFU/dt/ t_0, was chosen as 3 FU/min for this plot. See equation no. 2.

Figure 3 For each of nine points, medium was removed from D2 cells, the cells washed three times with 4mg/ml BSA in PBS and treated for
ten minutes with 50uM NPGB in PBS. The cells were then washed again in the same way and allowed to stand in the BSA solution for varying times before a 30 min assay with 0.5mg/ml plasminogen was performed.

(A) The mean value derived from the nine determinations of $k_3$ is plotted vs. $t_0$ which is the unknown difference in time between the removal of NPGB and the first appearance of a new PA. At $t_0 = 7$ min the mean value of $k_3$ is $1.9 \times 10^{-2}$ min$^{-1}$.

(B) The standard deviation of the nine experimental points plotted vs. $t_0$ shows a minimum of $0.2 \times 10^{-2}$ min$^{-1}$ at $t_0 = 7$ min.

**Figure 4** Empty micro wells were treated with 100ul of 200uM NPGB in PBS (2% DMF) for 5 min and quickly washed three times with 200ul aliquots of PBS. A fourth wash was allowed to stand in the micro well for the indicated time before it was replaced by 50ul of a trypsin solution for five minutes. The trypsin was then removed, titrated with FDE and the fluorescence measured. The control micro well was similarly treated but was not exposed to NPGB.

**Figure 5** Medium was removed from micro wells containing nearly confluent D2 cells or no cells and the micro wells washed once with PBS. At $t=0$, 250ul of 5uM FME plus 1ul NPGB-in PBS or 0.5 mg/ml Pmg in PBS were added to the micro wells. After the indicated time in the incubator, a 50ul aliquot was withdrawn and titrated with FDE. The fluorescence was then measured with a Perkin-Elmer 650-40. (A)
Plasminogen, FME and NPGB in an empty microwell and in one containing cells. (B) As in 5A but lacking plasminogen. (C) The difference between the curves in 5A, minus the difference between the curves in 5B, is plotted vs. time. The increase in fluorescence is presumably due to activation of plasminogen to plasmin.

**Figure 6** Fifty microliter aliquots of PBS containing 1mg/ml plasminogen, 2% DMF and varying NPGB concentrations were incubated over cells for two hours. One half of this activation was subjected to SDS PAGE (inset). A densitometer tracing of each slot was obtained and the areas of peaks corresponding to the heavy chain of plasmin (HC) determined. A peak area equal to \( \frac{1}{4} \) of the no-NPGB control occurs at about 5uM NPGB.

**Figure 7** Each sketch illustrates the results observed on a number of TLC plates. The origin in each sketch is near the lower left corner. From left to right is the first dimension of 200:3 H₂O:formic acid and from bottom to top is the second dimension of 9:1:1 benzene:pyridine:acetic acid. Fraction I and Fraction II plasminogenes seem to have about the same population of Glu and Lys amino-terminal species. Dog plasminogen apparently has a higher ratio of Lys to Glu than does human plasminogen.
### Table 1

<table>
<thead>
<tr>
<th>Pretreatments</th>
<th>Treatments</th>
<th>1st Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14 0.1M DTT in ...</td>
<td>0.1M Tris 50</td>
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<tr>
<td>PBS</td>
<td>22 PBS</td>
<td>15 PBS</td>
</tr>
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<td>10 0.1M EDTA</td>
<td>62 0.1M NaOH 104</td>
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<tr>
<td>0.1 M EGTA</td>
<td>14 0.1 M EGTA</td>
<td>40 4X PBS A 50</td>
</tr>
<tr>
<td>0.05M (EDTA+EGTA)</td>
<td>2 0.05M (EDTA+EGTA)</td>
<td>51 0.05M (EDTA+PEG) 77</td>
</tr>
<tr>
<td>L- Lysine</td>
<td>30 L- Lysine</td>
<td>26 NaN Lys A pH 7.2 2</td>
</tr>
<tr>
<td>0.1% Triton</td>
<td>44 0.1% Triton</td>
<td>81 0.1% Triton 22</td>
</tr>
<tr>
<td>4 M MIBA in PBS</td>
<td>2 Ethanol</td>
<td>1 Ethanol 65</td>
</tr>
<tr>
<td>Medium</td>
<td>20 - MIP</td>
<td>20 Medium 21</td>
</tr>
<tr>
<td>20% DMSO in PBS</td>
<td>33 10% DMSO</td>
<td>18 50% DMSO in PBS 78</td>
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<tr>
<td></td>
<td>0.1% Triton, pH 7.2</td>
<td>4 PBS, pH 5 7</td>
</tr>
<tr>
<td></td>
<td>NaN MIBA, pH 7.2</td>
<td>2 PBS, pH 3 71</td>
</tr>
<tr>
<td></td>
<td>NaN MIBA, pH 7.2</td>
<td>2 PBS, pH 3 90</td>
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<tr>
<td>Row</td>
<td>Treatment</td>
<td>PA Activity, % of Control</td>
</tr>
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<td>-----</td>
<td>----------------------------------</td>
<td>---------------------------</td>
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<tr>
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<td>Control</td>
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</tr>
<tr>
<td>2</td>
<td>30 min of DFP</td>
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</tr>
<tr>
<td>3</td>
<td>30 min of 'used' DFP</td>
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<tr>
<td>4</td>
<td>60 min of DFP</td>
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</tr>
<tr>
<td>5</td>
<td>30 min of DFP, 30 min of PBS</td>
<td>36</td>
</tr>
</tbody>
</table>
Decay of NPGB in Complete Medium

Figure 1

\[ \ln(\text{NPGB Remaining}) \]

TIME (min)
Inhibition of Pmg Activation by NPGB
FIGURE 7

Standards

Lysosome

Human Pug I

Human Pug II

Dog Pug I

Dog Pug II