

## PROTEASES PRODUCED BY NORMAL AND MALIGNANT CELLS IN CULTURE

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**Abstract:** The production of plasminogen activators (PA) is associated with the neoplastic transformation of many types of cells. A number of the phenotypic changes associated with transformation are dependent, at least in part, upon the generation of plasmin by the PA secreted by these cells. One of these changes, the loss of intracellular actin-containing cables in neoplastic cells, can be correlated with the level of extracellular proteases. Moreover, these same proteases can cause the disappearance of actin-containing cables in normal cells when supplied exogenously. Therefore, plasmin acting from the external side of the plasma membrane appears to be capable of causing the dissolution of these intracellular structures. The control of PA synthesis in two cell types, chick embryo fibroblasts infected with a temperature-sensitive mutant of Rous sarcoma virus and human embryonic lung cells, has been examined. Induction of PA requires both RNA and protein synthesis. Deinduction of PA also requires a new RNA and, perhaps, protein to be made. If the synthesis of this RNA is inhibited, PA production can continue for many hours under conditions that are normally non-permissive.

## INTRODUCTION

The association between increased fibrinolysis and oncogenic transformation has been demonstrated to occur in cultured fibroblasts from a number of different species (1-7). The increased fibrinolysis observed is the result of the

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activation of the zymogen plasminogen to its active form, plasmin, and the subsequent digestion of fibrinogen by plasmin (8). Plasminogen is a normal component of plasma and serum. The activation of plasminogen to plasmin is catalyzed by a second protease, plasminogen activator, that is produced by the transformed cells. The proteolytic nature of the activation can be demonstrated by monitoring the conversion of the single chain protein plasminogen to the two-chain enzyme plasmin by means of polyacrylamide gels (9). The plasminogen activators (PA) produced by malignant cells are active serine proteases with a trypsin-like specificity. They can be inhibited by DFP as well as other agents that react with proteases of this type. The PA produced by transformed cells of different animal species have molecular weights ranging from 45,000 for the enzyme from Rous sarcoma virus-transformed chick embryo fibroblasts to 72,000 for the PA from human melanoma cells (3,10).

A variety of experiments have indicated that the observed increase in production of PA by these cells is the result of transformation. Most normal fibroblasts have low levels of PA, but after transformation by oncogenic viruses or chemicals, the amount of PA increases dramatically (Table 1). A few nonmalignant cells, however, produce significant quantities of PA. These include human embryonic lung cells, human embryonic kidney cells, granular cells, activated macrophages and granulocytes (3,11,12). The increase in PA is not the result of viral infection since only transforming viruses induce the synthesis of PA (1); cytotoxic or temperate viruses do not induce such increases. Finally, cells infected with a mutant of RSV, which is temperature-sensitive for transformation, produce PA at the permissive temperature but not at the restrictive temperature (Fig. 1) (13). Thus, the production of PA appears to be a concomitant of transformation when fibroblasts are examined.

The production of PA has also been demonstrated to be related to the phenotype of the transformed cells. The ability of transformed cells to grow in agar (5,14), to migrate from the edge of a wound (15), and to express certain morphological changes (2,14,16) all appear to be dependent, at least in part, on the generation of plasmin. These observations, coupled with the reports that proteases may stimulate cell division (17-21) as well as alter the agglutinability of normal cells (22,23), indicate that the production of PA may have a significant effect on the in vitro properties of neoplastic cells.

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TABLE 1

Fibrinolytic activities of normal and transformed cells

Cell type	Radioactivity released into growth medium (% of total)
CEF	1.4
CEF-SR RSV	36.2
CEF-RAV50	1.4
HEF	1.4
HEF-SV40	31.0
MEF*	0.8
MEF-MSV*	70.0
HuEF	5.3
HuEL	75.4
Osteosarcoma*	44.5
Melanoma*	73.8
REF	5.1
REF-SV12	7.20
REF-SV9	61.5

The data were compiled from several papers and show the different fibrinolytic activities of cells or conditioned medium. Those experiments marked with an \* represent the activities found in serum-free medium collected from the designated cell type. All other experiments were performed by plating cells directly onto  $^{125}\text{I}$ -fibrin films and measuring the release of  $^{125}\text{I}$ . The abbreviations are: CEF, chick embryo fibroblast; CEF-SR RSV, chick embryo fibroblasts transformed by the Schmidt Rupp strain of Rous sarcoma virus; CEF-RAV50, chick embryo fibroblasts infected with avian leukosis virus; HEF, hamster embryo fibroblasts; HEF-SV40, hamster embryo fibroblasts transformed by SV40; MEF, mouse embryo fibroblasts; MEF-MSV, mouse embryo fibroblasts transformed by murine sarcoma virus; HuEF, human embryo

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fibroblasts; REF-SV12, a clone rat embryo fibroblast transformed by SV40; REF-SV9, a second clone of rat embryo fibroblasts transformed by SV40. Data taken from references 1, 2, 3, and 5.

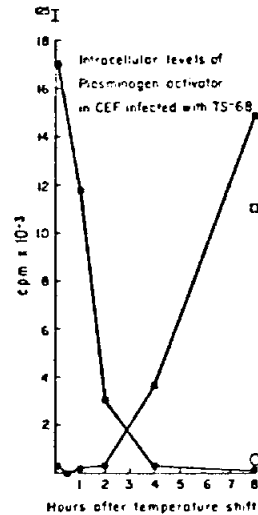


Fig. 1. Intracellular levels of PA in chick embryo fibroblasts infected with RSV ts 68. Chick embryo fibroblasts infected with RSV ts 68 were grown either at 41 or 36°C for at least one week, then trypsinized and plated at the appropriate temperature one day before initiation of the experiment. At zero time, the medium was removed from the cultures and replaced with fresh medium that had been warmed to the appropriate temperature; the cultures were then placed either at 36 or 41°C. At the indicated times, the medium was removed and the cells washed, scraped and centrifuged. The cell pellets were assayed for PA as described (13). ●—●, 36→41°C, RSV ts 68; ■—■, 41→36°C, RSV ts 68; ○, CEF, 41 or 36°C; □, RSV-SR-A, 41 or 36°C.

In this paper we have described experiments concerning two aspects of our studies on PA: the relationship between PA synthesis and intracellular actin organization as visualized by the use of antibodies to actin, and the mechanism of control of PA synthesis in normal and malignant cells.

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PLASMINOGEN ACTIVATOR AND CELL STRUCTURE

In an earlier report, a series of clones of SV40-transformed rat embryo fibroblasts (REF) were described (5). These clones varied over a tenfold range in their production of PA and produced between 40 and 300 times as much PA as did primary REF. When primary REF, cells from SVRE 12 (a clone that produces small amounts of PA), and cells from SVRE 9 (a clone that produces large amounts of PA) were examined by an indirect immunofluorescence procedure using anti-actin antibody, striking differences were apparent in the distribution and amount of actin-containing cables (Fig. 2) (24). The nontransformed cells (REF) exhibited many actin-containing cables. Conversely, SVRE 9 cells contained few actin-containing cables but did show a diffuse intracellular fluorescence. SVRE 12 cells had fewer actin-containing cables than REF cells but had considerably more structured actin than the SVRE 9 cells. Thus, there was an inverse correlation between the amount of PA synthesized by cells from each of these clones and the amount of polymerized actin they contained.

In most of the phenomena that have been examined, the effect of PA has been indirect and mediated through plasmin formation rather than directly through the action of PA. However, to produce significant levels of plasmin under tissue culture conditions, it is necessary to use a serum that is low in protease inhibitors and that contains a plasminogen easily activated by the PA produced. The studies illustrated in Fig. 2a-c employed fetal bovine serum which contains large amounts of protease inhibitors and a plasminogen poorly activated by rat PA. If the cells were grown in the presence of fetal bovine serum supplemented with dog serum, a serum that allows high levels of plasmin formation with rat PA, and the three cell types examined for the presence of actin-containing cables, a striking effect was detected (Fig. 2d-f). The REFs showed no difference in actin-containing cables dependent upon the serum supplement; cables were present under both conditions. Likewise, SVRE 9 cells exhibited little change in the amount of actin-containing cables as a function of the serum type. These two observations are consistent with the extremely low level of PA synthesis by REFs and the high level of synthesis of PA by SVRE 9. In the latter case, it is assumed that even in the presence of fetal calf serum enough plasmin is formed to dissociate the actin-containing cables. However, the SVRE 12 cells, which contained actin-containing cables when grown in the presence of fetal calf serum, were now found to be practically devoid of actin-

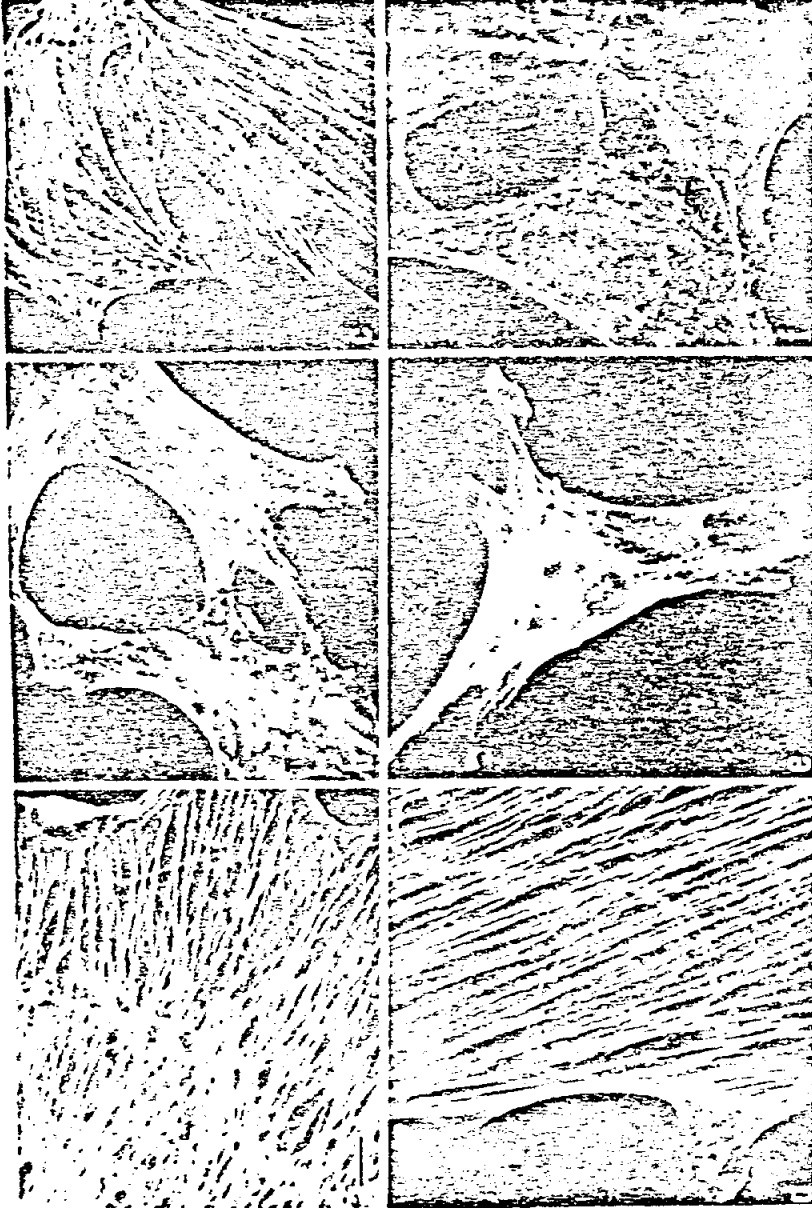


Fig. 2. Effect of serum species on actin cables in SV40-transformed and untransformed rat embryo cells. All cells plated on coverslips in Dulbecco's medium plus 10% fetal calf serum (a,b,c) or Dulbecco's medium plus 2.5% fetal calf serum plus 7.5% dog serum (d,e,f). The cell types examined were normal rat embryo cells (a,d), SVRE 9, (b,e), and SVRE12 (c,f).

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containing cables (Fig. 2). This can be explained by the higher fibrinolytic activity generated by these cells under these conditions.

These results are in agreement with the hypothesis that plasmin may be responsible for the loss of actin-containing cables in transformed cells producing PA. This hypothesis predicts that the actin-containing cables in normal REFs should be sensitive to externally supplied plasmin. This was tested by incubating rat embryo cells with plasmin generated from the combination of purified dog plasminogen plus urokinase, a PA purified from human urine. Cells were then scored for the presence or absence of cables as a function of time after the addition of plasmin (24). In cells exposed to plasmin there was a rapid loss of actin-containing cables within the first three hours (Fig. 3). Neither dog plasminogen nor urokinase by themselves had any effect on the

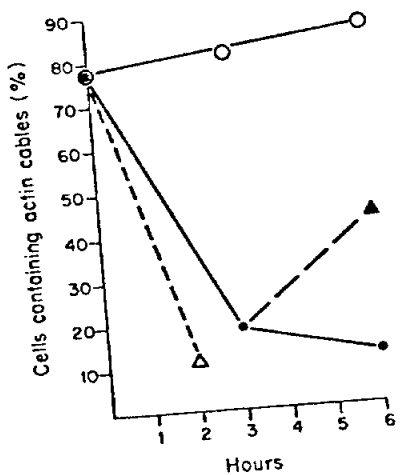


Fig. 3. Effect of proteases on actin-containing cables in REF. Rat embryo fibroblasts plated on coverslips were incubated with 1.5 μg/ml of purified dog plasminogen and 25 μg/ml of human urokinase. At the indicated times, coverslips were removed, the cells stained for actin-containing cables, and the percentage of cells with actin-containing cables was computed, ●-●. A control set of cultures received no protease, O-O. The proteolytic activity in a second set of plasmin-treated cultures was stopped by the addition of serum, Δ---Δ. A fourth set of cultures was treated with trypsin (2 μg/ml), Δ---Δ.

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percentage of cells seen to have actin-containing cables. Similar results were obtained if the cells were exposed to purified trypsin rather than plasmin. Two other proteases, chymotrypsin and thrombin, were also able to dissociate actin-containing cables but much less efficiently than either plasmin or trypsin (24). If the activity of plasmin was arrested by the addition of fetal calf serum to the cultures, there was a subsequent reappearance of actin-containing cables indicating that the cells retained the ability to polymerize actin (24).

Thus, it appears that extracellular proteases can affect the internal structure of cells in culture. It is interesting to speculate on the general role that such proteolytic activities may play in the normal shape changes seen in mitosis. It is also of some interest to ask how such enzymes, presumably acting externally to the plasma membrane, are able to affect internal structures.

## CONTROL OF PA SYNTHESIS

To study the control of PA synthesis, we chose to examine the macromolecular requirements for PA production in chick embryo fibroblasts infected with a mutant of Rous sarcoma virus temperature-sensitive for transformation (25). Earlier studies had shown that cells infected with viruses of this type produced PA at the permissive temperature, 36°, but not at the restrictive temperature, 41° (1). In Fig. 1 are shown the results of an experiment employing CEF-TS 68 cells shifted from either permissive to restrictive or restrictive to permissive temperatures and assayed for intracellular PA. In cells shifted from 41° to 36°, the enzyme appeared rapidly and could first be detected between 2-3 hours. This initial production of PA could be prevented by the inclusion of inhibitors of RNA and protein synthesis in the culture medium at the time of temperature shift (13).

The deinduction of PA synthesis upon shift of cells from 36° to 41° was extremely rapid, with a half time of less than one hour (Fig. 1). This was unexpected since earlier experiments had indicated that PA was the product of a cellular gene and would not be expected to be temperature-sensitive in these cells. Experiments (not illustrated) indicated that, indeed, the PA produced by CEF-TS68 was not significantly temperature-labile (13). However, a striking result was observed when the effects of inhibitors of macromolecule synthesis on PA deinduction were examined. Although the

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inhibition of protein synthesis by the addition of cycloheximide at the time of temperature shift resulted in the accelerated loss of PA activity, the inhibition of DNA-dependent RNA synthesis by the addition of actinomycin protected against the loss of PA activity (Fig. 4). Cells shifted to the restrictive temperature in the presence of actinomycin contained high levels of PA for periods up to 18 hours, at which time the state of the cells had deteriorated to such a degree that the experiment was terminated.

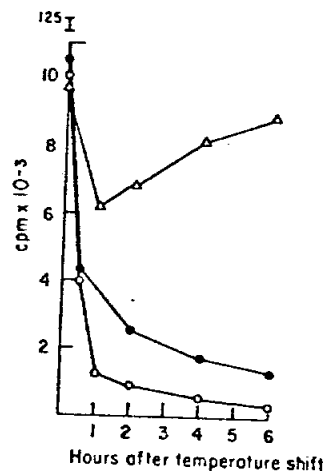


Fig. 4. The effect of inhibitors of macromolecule synthesis on PA levels in chick embryo fibroblasts infected with ts 68. Chick embryo fibroblasts infected with ts 68 were grown at 36°C. At zero time, the culture medium was removed and replaced with medium warmed to 41°C. One third of the cultures received medium containing actinomycin (1 μg/ml); one third, medium containing cycloheximide (20 μg/ml); and one third, medium alone (controls). The cultures were then placed at 41°C, and at the appropriate times, the cells were scraped and assayed for PA as described in the text. ●—●, control; Δ—Δ, actinomycin (1 μg/ml); ○—○, cycloheximide (20 μg/ml).

The nature of the requirement for the inhibition of RNA synthesis to prevent deinduction was added at progressively later times after the temperature shift and the final level

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of PA observed (Table 2). The ultimate steady state value of PA was dependent upon the time of addition of the drug. If the actinomycin was added later than two hours after the temperature shift, there was little retention of PA. These results also demonstrate that the ability of these cells to synthesize PA cannot be recovered at 41° if RNA synthesis is inhibited too late after temperature shift.

TABLE 2

Protective effect of actinomycin on PA synthesis as a function of time of addition after temperature shift

Time of addition of actinomycin (hr)	Final level of PA (% control at 0 time)
0	75
1	39
2	24
-	9.8

Chick embryo fibroblasts infected with TS68 were grown at 36°. At the initiation of the experiment, cultures were shifted to 41°. At the times indicated, actinomycin (1 µg/ml) was added to the cultures. Eight hours after the initiation of the experiment, the cultures were scraped and assayed for PA.

In the second experiment, the question asked was whether the requirement for the inhibition of RNA synthesis upon temperature shift was continuous or transitory. In this experiment, the drug 5-bromotubercidin (BuTu) was used in place of actinomycin. Earlier experiments had indicated that BuTu, like actinomycin, inhibited DNA-dependent RNA synthesis but, unlike actinomycin, the effect of BuTu was reversible (25). Thus, in cultures of CEF-TS68 shifted to 41° in the presence of BuTu (Fig. 5), PA synthesis was observed to continue. In comparison cultures, the BuTu was removed 2 or 5 hours after the shift to 41° had taken place. The level of PA immediately fell coincidental with the resumption of RNA synthesis. Thus, it appears that the continuous suppression of RNA synthesis is required to permit the production of PA at the non-permissive temperature.

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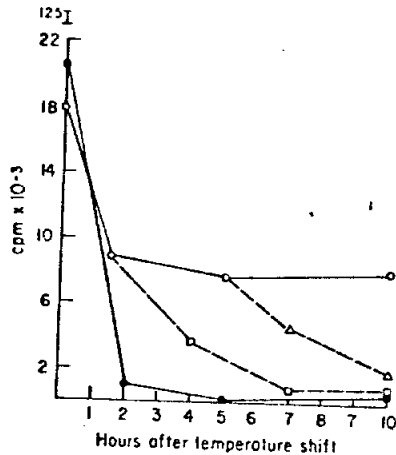


Fig. 5. The reversible effect of bromotubercidin. Cultures of chick embryo fibroblasts infected with TS68 were treated precisely as described in the experiments illustrated in Fig. 1. However, at 2 and 5 hours after the temperature shift, the bromotubercidin was removed from some sets of cultures and replaced with normal medium. ●—●, Control; ○—○, bromotubercidin (10 µg/ml); □---□, bromotubercidin washed out at 2 hr; △---△, bromotubercidin washed out at 5 hr.

The maintenance of high levels of PA in cells held at the nonpermissive temperature in the presence of actinomycin could represent either de novo enzyme synthesis or the retention of enzyme synthesized at 36°. Since, as yet, techniques are not available to measure directly de novo synthesis of PA, we have attempted to demonstrate continued enzyme synthesis by several indirect methods. For example, if protein synthesis is inhibited by the addition of drugs such as cycloheximide to cultures previously treated with actinomycin at the time of temperature shift, there is an immediate loss of PA activity (results not shown) (13). The decrease in PA is coincident with the inhibition of protein synthesis. A second approach to the question of de novo enzyme synthesis is illustrated in Table 3. Cells infected with TS68 were shifted from 36° to 41° in the presence of both actinomycin and cycloheximide. After the intracellular PA content had fallen to less than 10% of the initial value, the cycloheximide was removed and protein synthesis allowed to resume. A rapid increase in the intracellular content of PA was then observed. Thus, cells containing low levels of PA were able to re-establish high levels of PA upon the reinitiation of protein

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TABLE 3

Reversibility of cycloheximide inhibition of PA synthesis

Conditions at 41°	PA (% of actinomycin treated)
Actinomycin at time of shift	100
No drug at time of shift	5
Actinomycin + cycloheximide at time of shift	16
Actinomycin + cycloheximide at time of shift; cycloheximide removed at 2 hr.	98

Chick embryo fibroblasts infected with TS68 and grown at 36° were shifted to 41° and the drugs indicated were added to replicate cultures. Actinomycin was used at 1 µg/ml and cycloheximide at 2 µg/ml. At the end of 8 hr, all cultures were scraped and the amounts of PA determined.

synthesis. Finally, an experiment was performed to measure both the intracellular and extracellular levels of PA in control and actinomycin-treated cultures after shift from 36° to 41° (Table 4). Cultures that had received actinomycin continued to secrete PA into the culture medium at linear rates for periods up to 16 hours while simultaneously retaining high intracellular levels of PA. In control, nondrug-treated cultures, the extracellular level initially rose sharply but then leveled off in concert with the decrease in intracellular PA. The retention of a high intracellular level of PA with the continued secretion of PA into the culture medium, as well as the results from the other experiments, is most easily interpreted as the result of continued synthesis of PA at 41°. However, proof of *de novo* synthesis will require the isolation of enzyme biosynthetically labeled with radioisotopes added to the culture after the temperature shift.

Although the synthesis of a new RNA appeared to be required for deinduction of PA upon temperature shift, the question of whether a similar requirement existed for protein synthesis during deinduction remained unanswered. This was approached in the following manner. Cells (CEF-TS68) were shifted from 36° to 41° in the presence of cycloheximide. RNA synthesis was allowed to take place under these conditions for 4 hours. The cultures were then washed free of cycloheximide to permit the resumption of protein synthesis and exposed to actinomycin to inhibit RNA synthesis. If RNA

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TABLE 4

## Intra- and extracellular levels of PA

Additions		PA (% of actinomycin treated)
<u>A. Intracellular activity</u>		
-	3 hr	12.9
	6 hr	10.2
	16 hr	10.3
Actinomycin (1 $\mu$ g/ml)	3 hr	100
	6 hr	100
	16 hr	100
		PA activity cpm x 10 <sup>3</sup>
<u>B. Extracellular activity</u>		
-	3 hr	7.4
	6 hr	10.2
	16 hr	12.0
Actinomycin	3 hr	10.4
	6 hr	17.6
	16 hr	30.4

Chick embryo fibroblasts infected with TS68 and grown at 36° were shifted to 41°. Actinomycin (1  $\mu$ g/ml) was added to half the cultures. At the times indicated, cultures were scraped and assayed for PA. To assay extracellular activity the medium was removed from one set of cultures and assayed for PA.

synthesis by itself was sufficient for deinduction, there should have been no resumption of PA activity. If both protein and RNA synthesis were required, then PA content should have increased. Table 5 shows that PA level did increase under these conditions, a result consistent with an assumed requirement for both RNA and protein synthesis for PA deinduction. This result also implies that the new RNA synthesized must turn over rapidly since it has little effect once protein synthesis is reinitiated in the absence of continued

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RNA synthesis.

TABLE 5

Requirement for protein synthesis for PA deinduction

Additions	PA (% of actinomycin treated)
-	5
Actinomycin (1 $\mu$ g/ml)	100
Cycloheximide (2 $\mu$ g/ml)	10
Cycloheximide (2 $\mu$ g/ml); remove cycloheximide at 4 hr and replace by actinomycin (1 $\mu$ g/ml)	92

Chick embryo fibroblasts infected with TS68 and incubated at 36° were shifted to 41° in the presence of the indicated drugs. After 4 hr at 41°, the cycloheximide was removed from one culture and replaced by actinomycin. At the end of 8 hr, the cultures were scraped and assayed for PA.

Results similar to these have been observed when the deinduction of several enzymes found in eukaryotic cells has been perturbed by the addition of drugs such as actinomycin (27). Tomkins has presented a model to explain these observations in which deinduction of the enzyme in question requires the synthesis of a new species of RNA (28). The presence of actinomycin at the time of shift from permissive to restrictive conditions prevents the synthesis of this RNA and thus the production of enzyme continues from a stable mRNA. The exact mechanism by which this RNA initiates deinduction has not been elucidated, nor has the RNA been isolated.

Our results are entirely consistent with this model. It should be noted, however, that Tomkins's proposal has been criticized by several workers (29) and that alternative models can be proposed which fit the data equally well (see Discussion). The resolution of this problem will require a more careful analysis, including the isolation of the controlling molecules.

A normal cell found to produce PA is the human embryonic lung cell (HuEL) (3). These cells generate PA at rates comparable to those seen by malignant cells. When HuEL cells

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are exposed to corticosteroids, there is a rapid inhibition of PA synthesis (Table 6). Steroids of other classes are not

TABLE 6

Effect of steroids on PA synthesis in human embryonic lung cells

Steroid addition	Activity (% of control)
-	100
Dexamethasone	10.9
Fludrocortisone	9.7
Hydrocortisone	7.9
Corticosterone	17.7
Prednisolone	25.4
Cortisone	41.2
Progesterone	69.4
<sup>4</sup> -Androsten-3,7-dione	75.3
Testosterone	96.5

Human embryonic lung cells plated at  $5 \times 10^5$  cells per 60-mm dish were exposed to the indicated hormones ( $10^{-6}$  M) overnight. The next day the cells were scraped and assayed for PA.

effective. The ability of corticosteroids to cause the de-induction of PA in HuEL cells is of potential medical interest because of the proposed involvement of both corticosteroid levels and the fibrinolytic system in respiratory disease syndrome (RDS) (30). Children with this disease have an impaired fibrinolytic system that manifests itself by the inability of the infant to remove the fibrin-containing hyaline membrane that forms in their lungs at birth. This may be due to a lack of plasminogen resulting from premature conversion of plasminogen to plasmin by the PA from HuEL cells. The low levels of corticosteroids in children with RDS suggest that abnormal PA production may occur in these infants (31). The reported beneficial effects of corticosteroid therapy in preventing RDS is consistent with this hypothesis. More experiments are necessary, however, to document this proposed

relationship between the PA from HuEL and RDS.

The ability to modulate with corticosteroids PA production in these cells has permitted an analysis of PA deinduction in this system and a comparison with that seen in CEF-TS68 cells upon temperature shift. No deinduction was observed when actinomycin was added at the same time as dexamethasone (Fig. 6). This effect has also been observed with

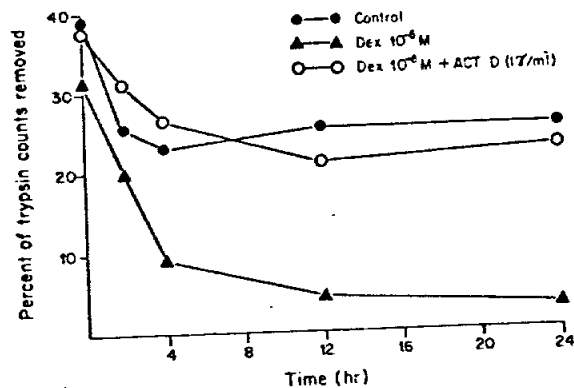


Fig. 6. Effect of dexamethasone on PA synthesis by human embryonic lung cells. Human embryonic lung cells were incubated with  $10^{-6}$  M dexamethasone,  $\blacktriangle$ — $\blacktriangle$ ,  $10^{-6}$  M dexamethasone + actinomycin ( $1 \mu\text{g}/\text{ml}$ ),  $\circ$ — $\circ$ , or Eagle's medium alone,  $\bullet$ — $\bullet$ . At the indicated times, cells were scraped and assayed for PA.

PA deinduction by corticosteroids in other cell systems (32, 33). Other experiments have indicated that the requirements for PA deinduction in HuEL cells are similar to those found for CEF-TS68 with one exception. There appears to be no requirement for protein synthesis to initiate deinduction. This may indicate an intrinsic difference in the two cell types, or it may be due to the less responsible nature of the deinductive process in HuEL cells.

#### CONCLUSION

In this paper we have attempted to demonstrate how the production of a particular component of the fibrinolytic system may be implicated in the etiology of diverse pathological conditions. The biological role of PA appears to be extensive, with possible involvement in processes such as ovulation,

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cell growth, cell shape, neoplasia, as well as hemostasis. Thus, the study of PA in these various processes promises to yield information concerning the basic nature of a number of biological phenomena.

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