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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THE PLASMINOGEN ACTIVATOR IN TERATOCARCINOMA STEM CELLS INDUCED WITH SODIUM BUTYRATE

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SUMMARY Suspension cultures of pluripotent teratocarcinoma cells were induced with sodium butyrate to produce plasminogen activator (PA), generally regarded as a marker enzyme of differentiation of the teratocarcinoma. The induction of plasminogen activator was very efficient, resulting in production of sufficient enzyme to allow its purification. The activator was inactivated by rabbit anti-human melanoma plasminogen activator antiserum, indicating that it was a tissue-type activator (t-PA).

The enzyme was purified by column chromatographus on phosphocellulose, zinc-chelate agarose, Con-A Sepharose and Sephadex G-150. The preparation at the final step of purification gave a single peak of enzyme activity at pH 7.3 ± 0.1 on isoelectric focusing, and showed a molecular weight of $\sim$77,000 on SDS PAGE.

INTRODUCTION

Serine protease plasminogen activator is generally accepted to be a marker of endoderm cells, and may play an important role in embryogenesis; the plasminogen activator functions in the interaction between cells and their surrounding matrix and in cellular migration (Strickland et al., 1976).

Plasminogen activators from various sources have been classified into two major groups, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), based on their immunological properties. In a recent report on the distribution on plasminogen activators (u-PA and t-PA) in early mouse embryonic cells it was proposed that one type of enzyme (u-PA) is produced by both visceral endoderm and extraembryonic mesoderm, while the other type (t-PA) is synthesized by parietal endoderm (Marotti et al., 1982). These reports prompted us to purify plasminogen activator to obtain a understanding of its character and role in mammalian embryogenesis.

Mouse teratocarcinoma cells can differentiate in vivo and in vitro (Matin and Evans, 1975), producing a variety of cell types, mi-

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micking normal embryogenesis and so providing a good system for use in studies on the determination and differentiation of mammalian cells under controlled conditions. Chemicals, such as retinoic acid and dibutyryl cyclic AMP (Strickland and Mahdavi, 1978), or DMA and polybrene (Speers et al., 1979) are known to inducer teratocarcinoma cell differentiation. Recently we demonstrated that the pluripotential teratocarcinoma cell line 311 was induced to produce plasminogen activator much more effectively with sodium butyrate than with other drugs (Nishimune et al., 1983).

Here we report the establishment of a simple system for mass culture of teratocarcinoma cells to produce a large amount of plasminogen activator, and the purification and characterization of plasminogen activator produced on induction with sodium butyrate.

MATERIALS AND METHODS

1. *Materials*

The following reagents were used: sodium butyrate, immidazole, polyethylene glycol, Triton X-100 and Tween 80 (Wako Pure Chemical Industries, Ltd.); fibrinogen, potassium thiocyanate and acrylamide (Nakarai Chemicals, Ltd.); zinc-chelate agarose, Con-A Sepharose and Sephadex (Pharmacia Fine Chemicals); retinoic acid (all trans) and Coomassie brilliant blue G-250 (Sigma Chemical Co.); lactoalbumin hydrolysate and gelatin (Difco Laboratories); human urokinase and thrombin (Green Cross Co.); bovine plasminogen (Daiichi Chemicals); phospho-cellulose (Whatman); Aprotinin (Boehringer Mannheim Yamanouchi); α-D-methylmannoside (Fluka A.G.); sodium dodecyl sulfate (SDS) (Pierce); ampholine (LKB); pI markers (Oriental Yeast Ltd.).

2. *Cell line*

The pluripotent teratocarcinoma cell line 311 (Ogiso et al., 1980), was used throughout. The stock culture was passaged on a feeder layer twice weekly in Eagle's MEM containing 5 m*M* glutamine, 1 m*M* sodium pyruvate and 10% fetal calf serum (FCS) at 37 C in a humidified atmosphere of 5% CO_2 in air. The feeder layer was a confluent monolayer of BALB-A31 cells treated with 10 μg/ml of mitomycin C for 3 h. For experiments, the stock culture was treated with 0.125% trypsin and 0.5 m*M* EDTA in phosphate buffered saline (PBS) at 37 C for 10 min and then the cells were seeded at 10^6 cells per 60 mm gelatin coated culture dich previsouly incubated with fetal calf serum (0.2 ml/60 mm dish) in medium supplemented with 10^{-4} *M* β-mercaptoethanol (Oshima, 1978).

3. *Formation of tumors and preparation of plasminogen activator in culture*

Teratocarcinoma 311 cells, that had been cultivated in vitro were injected subcutaneously (5×10^6 cells) into both sides of the inguinal region of 129/sv mice. About 20 days after the injection, tumors were excised aseptically, washed with PBS, and cut into fragments (1–2 mm) in PBS containing 1 m*M* EDTA. Those fragments were treated with 0.05% trypsin and 0.83 m*M* EDTA in PBS for 10 min at 37 C with stirring, and after addition of bovine serum the preparation was centrifuged. The pellet was suspended in Dulbecco's modified Eagle's medium containing 0.1% lactoalbumin hydrolysate, 5 m*M* glutamine and 10% FCS and stood for 20 min at 37 C. Then the preparation was centrifuged and the pellet was suspended in the same medium as before but without serum in siliconized glass flasks. Suspension cultures were initiated with cells from about ten tumors ($\sim\times10^9$ cells) in 1-liter of serum-free medium in 3-liter siliconized glass flasks. After the addition of sodium butyrate (5 m*M*–10 m*M*) as an inducer of teratocarcinoma cell differentiaion, the flasks are shaken at 60 strokes/min at 37 C. After culture for 1–2 days, the culture medium was centrifuged and the supernatant was stocked. The pellet was resuspended in fresh medium containing inducer and cultured again. This process was repeated two to three times and the culture fluids obtained were stocked as a source of plasminogen activator.

4. *Assay of plasminogen activator*

Plasminogen activator was assayed by measuring plasminogen dependent fibrinolysis on plasminogen-rich and free fibrin plates (Astrup and Mullertz, 1952; Kwaan and Astrup, 1976). For this, plasminogen-rich and free fibrinogen solutions (3.2 mg/0.4 ml of 0.1 *M* phosphote buffer at pH 7.2) were mixed with thrombin solution (4 U/0.4 ml of 0.1 *M* phosphate buffer at pH 7.2) in ice water

and the mixtures were quickly plated on slide glasses (26 mm×76 mm). Drops of 10 μl of sample solution were applied to the slides and incubated in a humid chamber at 37 C. After 24 h, the area of the zone of lysis was measured and plasminogen-dependent fibrinolysis was confirmed by the absence of lysis on a slide of plasminogen-free fibrinogen. The area of the zone of lysis by the sample was compared with that by standard solution of urokinase and the activity was expressed in International Units (IU).

5. *Inhibition of plasminogen activator by anti-tissue activator antiserum*

Rabbit anti-serum was raised against human melanoma plasminogen activator (a tissue-type activator), kindly provided by Kanegafuchi Chamical Co. Concentrated solutions of plasminogen activator from human melanoma, mouse teratocarcinoma and human urokinase were incubated for 90 min at room temperature with increasing amounts of anti-human melanoma plasminogen activator antiserum. Then, aliquots of the mixtures were assayed for the residual activity of plasminogen activator by the fibrin plate method.

6. *Purification procedure*

Purification was achieved by the method of Rijken and Collen (1981), with the slight modifications that phospho-cellulose column chromatography was used for concentration of culture fluid and the eluate with 0.5 *M* NaCl was applied to affinity column chromatographies on zinc-chelate agarose and Con-A agarose followed by gel filtration on Sephadex G-150. Protein concentration was measured by the method of Lowry et al. (1951), with bovine serum albumin as a standard.

7. *SDS polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing*

The separation gel contained 10% polyacrylamide was used and gels were stained with Coomassie brilliant blue G-250 and sliver stain (Bio-Rad Sliver Stain). Molecular weight was determined with a low molecular weight calibration kit (Pharmacia, Uppsala, Sweden). Isoelectric focusing was performed in 4% polyacrylamide gel at 200 V at 4 C for 7 h in a glass tube (14 cm×0.5 cm) with LKB ampholines (pH range 3.5–10). The gels were shaken gently in 62.5 m*M* Tris-HCl buffer (pH 6.8) to remove the ampholines and cut into 5 mm slices. Each slice was homogenized in 20 m*M* phosphate buffer (pH 7.2), and stood overnight at 4 C and the supernatants were assyed on fibrin plates. The colored marker proteins were subjected to soelectric focusing under the same conditions for comparison with samples.

8. *Zymographic analysis*

Zymographic analysis was carried out as described by Heussen and Dowdle (1980). Briefly, electrophoresis in polyacrylamide gel containing 0.1% gelatin, bovine plasminogen and 0.1% SDS was performed at 4 C at a constant current of 8 mA until the BPB marker reached the bottom of the gel. The gels were then washed with 2.5% Triton X-100 to remove SDS, incubated in glycine-NaOH buffer (pH 8.3) for 3–5 h at 37 C and stained with Coomassie brilliant blue.

RESULTS

1. *Induction of plasminogen activator with sodium butyrate in the pluripotent teratocarcinoma cell line 311*

A large number of teratocarcinoma cells are required for production of sufficient plasminogen activator for its purification. These can be obtained by subcutaneous cultivation of teratocarcinoma stem cells in vivo. Teratocarcinoma cells were prepared as described under Materials and Methods. The effect of sodium butyrate on production of plasminogen activator by the cells in vitro is showen in Fig. 1. The induction was greatest with 5 m*M*–10 m*M* sodium butyrate. Accumulation of plasminogen activator in the medium is shown in Fig. 2. Plasminogen activator was detectable after 10 h and increase in its activity continued until at least 22 h and then stopped. For at least three times, medium change resulted in production of the same amount of plasminogen activator again. The production of plasminogen activator by cells in suspension culture was similar to that of cells cultured on plastic tissue culture dishes (date not shown). On the contrary, no detectable activator was produced until at least 94 h on treatment of the suspension culture with 10^{-6} M retinoic acid (a well-known inducer).

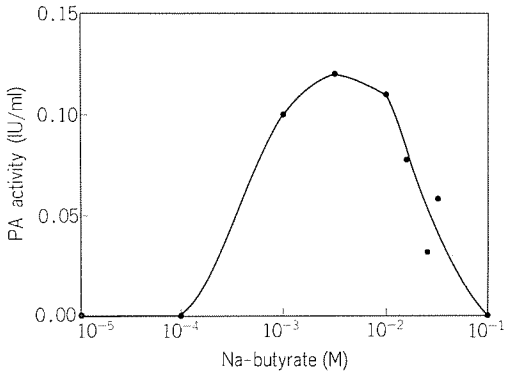

FIGURE 1. Effect of concentration of sodium butyrate in the culture medium on production of plasminogen activator.
Teratocarcinoma cells at a concentration of 10^5 cells/ml were incubated in medium containing an appropriate concentration of sodium butyrate in agar-coated dishes to keep the cells in suspension. After 48 h, the culture fluid were collected and centrifuged. The supernatants were assayed for plasminogen activator. Abscissa: Concentration of sodium butyrate in medium. Ordinate: Plasminogen activator activity (IU/ml).

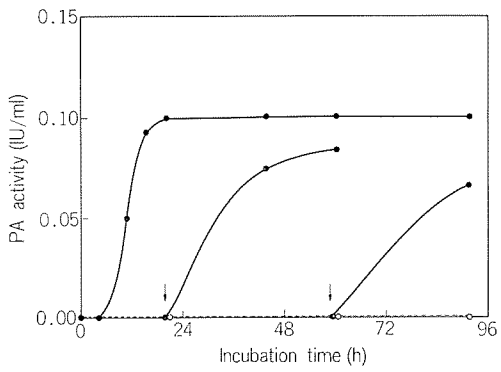

FIGURE 2. Effects of incubation time and medium change on production of plasminogen activator.
Teratocarcinoma 311 cells from tumors are incubated as described under Materials and Methods and treated with sodium butyrate (●) or retinoic acid (○). At appropriate times, plasminogen activator in aliquots of culture fluid was assayed. Medium in some flasks was changed at 22 h and 60 h (↓). Abscissa: Incubation time in hours. Ordinate: Plasminogen activator activity (IU/ml).

These results indicated that the suspension culture produces plasminogen activator fairly efficiently and that sodium butyrate is a better inducer than retinoic acid.

2. *Inhibition of plasminogen activator by anti-tissue activator antiserum*

Tissue-type plasminogen activator differs immunologically from the urokinase-type; anti-tissue-type activator antiserum was reported to inhibit tissue-type but not urokinase-type plasminogen activator (Marotti et al., 1982; Rijken and collen, 1981). Therefore, we examined the inactivation of plasminogen activator in the culture fluids of human melanoma and mouse teratocarcinoma cells with antihuman melanoma plasminogen activator antiserum. For this, after incubation of of culture fluids with an appropriate amount of antiserum, residual activity of plasminogen activator was assayed. Although the plasminogen activator of mouse teratocarcinoma cells seemed to be less sensitive than human melanoma plasminogen activator to the antiserum, the inactivation curve of the two were similar (Fig. 3). These results indicated that plasminogen activator in the culture fluid of mouse teratocarcinoma cells was tissue-type plasminogen activator.

3. *Purification and characterization of plasminogen activator*

About 18 liters of culture fluid of teratocarcinoma 311 cells, obtained as described under Materials and Methods, was used as starting material. It had a protein content of about 1.0 mg/ml and an activity of about 0.1 IU/ml. All purification procedure were performed at 4 C in media containing Tween-80 and aprotinin except the last gel filtration and isoelectric focusing steps. On a phosphocellulose column, the plasminogen activator was concentrated about 10 times and most of the lactoalbumin hydrolysate present in the medium was removed. Because the plasminogen activator secreted into the culture fluid

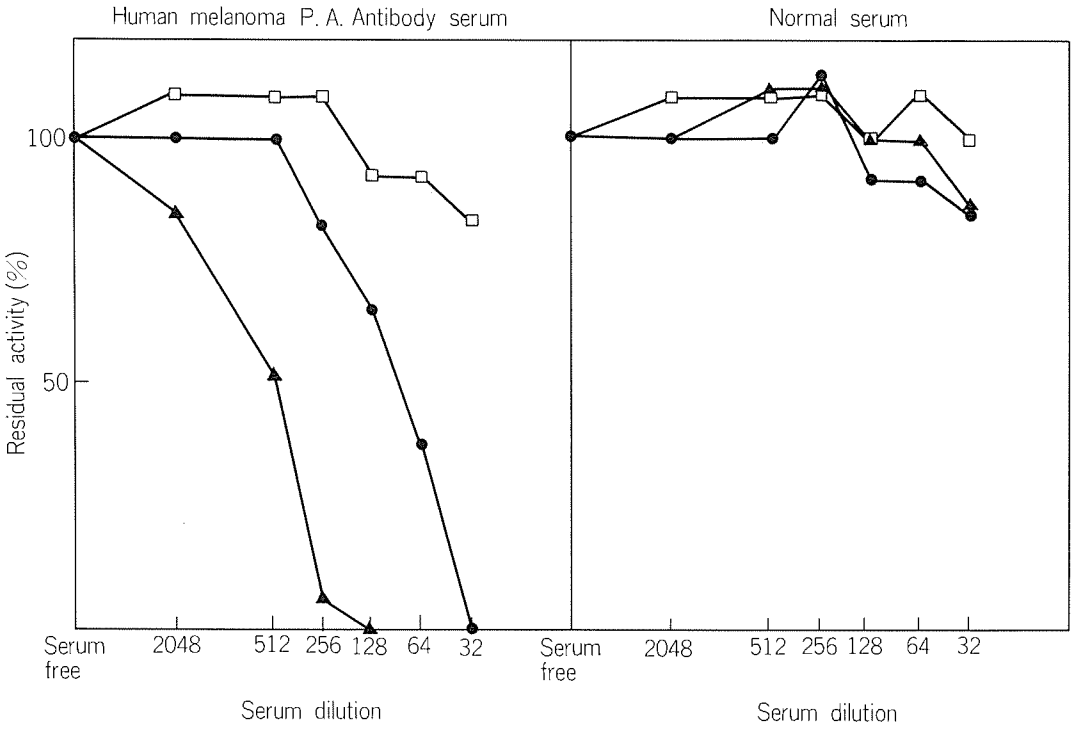

FIGURE 3. Inhibition of plasminogen activator by anti-tissue activator antiserum. Volumes of 15 μl of human urokinase (□), human melanoma plasminogen activator (▲) and mouse teratocarcinoma plasminogen activator (●) were incubated with serial dilutions of antihuman melanoma plasminogen activator antiserum (15 μl) as described under Materials and Methods. Residual activities (ordinate) expressed as percentages of those without serum are plotted against the dilution of the serum (abscissa). 1) Rabbit anti-human melanoma plasminogen activator antiserum. b) Normal rabbit serum.

TABLE 1. *Purification of the plasminogen activator from the culture fluid of mouse teratocarcinoma cell line 311.*

The data were obtained during purification of 18,000 ml of culture fluid. Details of experiment procedures are described in the Methods

	Volume (ml)	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Yield (%)	Purification factor
Conditioned Medium	18,000	18,450	1,800	0.1	100	1
P-Cellulose	1,400	340	765	2.2	42	22
Zn-chelate agarose	95	16.5	228	13.9	13	139
Con A-sepharose	110	12	228	19.0	13	192
Sephadex G-150	2.5	0.125	50	400	3	4,000

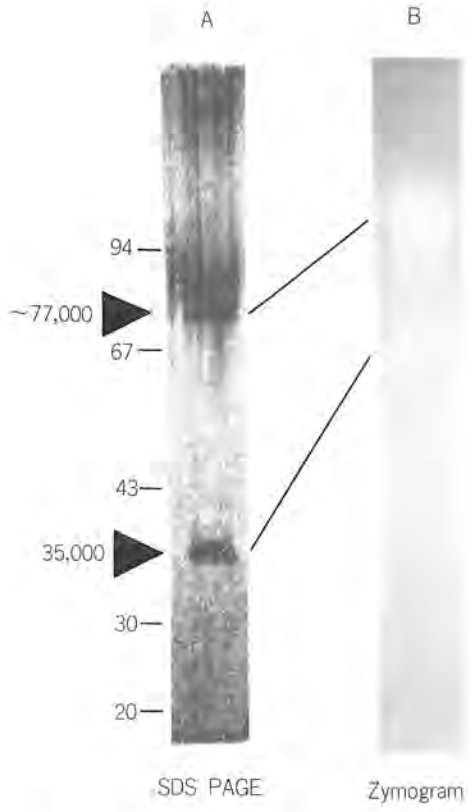

FIGURE 4. SDS-Polyacrylamide gel electrophoresis (PAGE) and zymographic analysis of purified plasminogen activator. A: SDS ploy acrylamide gel electrophoresis of purified plasminogen activator (eluate from Sephadex G-150 column) with silver staining. The positions of molecular weight markers run in parallel are indicated. B: Zymogram of purified plasminogen activator. Gelatin hydrolyzed by plasmin activated by plasminogen activator is not stained with Coomassie brilliant blue and appears as transparent bands, demonstrating the position of plasminogen activator.

of teratocarcinoma cells was the tissue-type, as described before, we used a modification of the method for purification of plasminogen activator produced by melanoma cells (Rijken and Collen, 1981). A zinc-chelator column was used for affinity chromatography and plasminogen activator was eluted with about 0.02 *M* immidazole as a single peak. Then the preparation was subjected to Concanavaline-A Sepharose column chromatography and enzyme activity was eluted with about 0.2 *M* α-D-methylmannoside containing 1 *M* potassium thiocyanate and subjected to gel filtration on Sephadex G-150 (superfine) without treatment with the protease inhibitor, aprotinin. The result of the purification procedure are summarized in Table 1. The recovery of enzyme activity was 3% and the specific activity of the finally preparation was about 4,000 times that of the starting material. On SDS-gel electrophoresis, the purified plasminogen activator fraction gave two bands of material with molecular weights of ~77,000 and 35,000 (Fig. 4A). Although both material showed enzyme activity on zymographic analysis (Fig. 4B), the specific activity of the material with a molecular weight of ~77,000 was higher than that of the 35,000 protein. On isoelectric focusing, the purified plasminogen activator fraction gave a single peak of enzyme activity at pH 7.3±0.1 (Fig. 5). Moreover, the protein in this peak showed a molecular weight of ~77,000 on SDS PAGE (Fig. 5).

DISCUSSION

Plasminogen activator is often used as a marker in studies on differentiation of teratocarcinoma cells in vitro (Sherman et al., 1976; Topp et al., 1976). However, biochemical characterization of this enzyme has been difficult, because insufficient material is obtained under normal culture conditions. We established a simple new method to obtain a large amount of plasminogen activator in the culture fluid of pluripotent teratocarcinoma cells from tumors induced with sodium butyrate. This system is useful for obtaining a large number of cells without using culture dishes and obtaining a large amount of plasminogen activator. Since the effect of sodium butyrate on plasminogen activator production appeared faster than that of retinoic acid (a well-known inducer, Strickland and Mahdavi, 1978), this

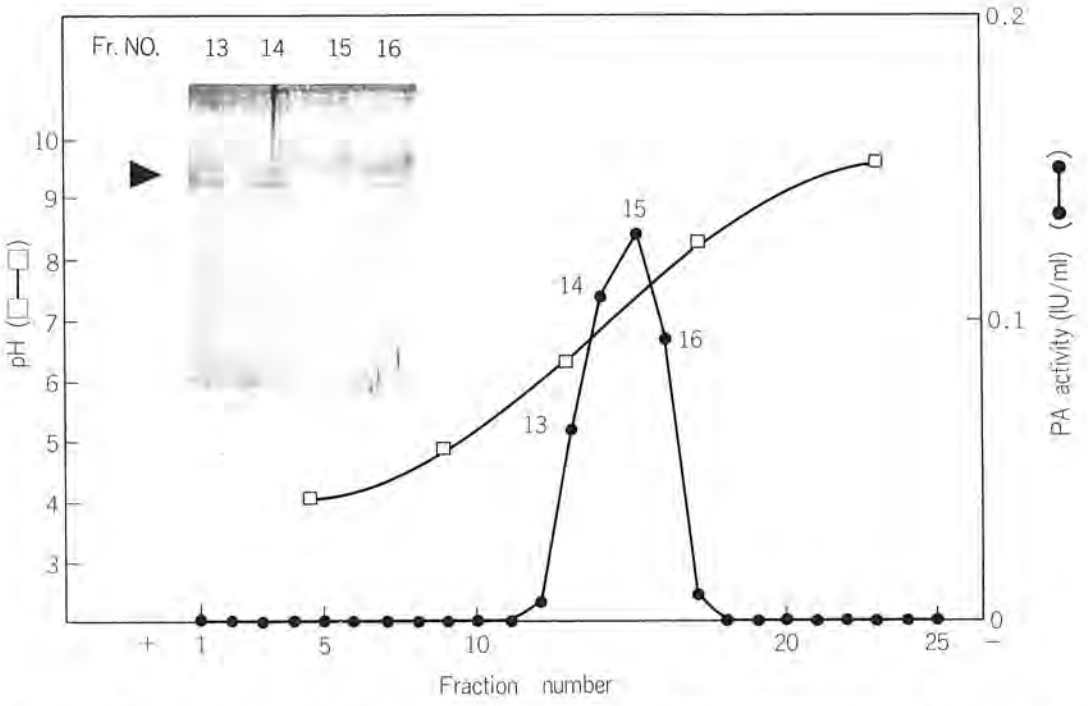

FIGURE 5. Isoelectric focusing of purified plasminogen activator. After isoelectric focusing, plasminogen activator was eluted from sliced gels and its activity was assayed (●). The positions of PI marker proteins, cytochrome c and acetylated cytochrome c (pH 9.7, 8.3, 6.4 4.9, and 4.1), are plotted (□). Inset: SDS-PAGE of peak fractions (Fr. 13, 14, 15, 16) of plasminogen activator stained with silver.

compound is better for use on mass cultures of teractocarcinoma cells. We purified the plasminogen activator and found that it had molecular weights of ~77,000 and 35,000 on SDS-polyacrylamide gel electrophoresis (Fig. 4A). Wallen et al (1981) reported that two chains of tissue type plasminogen activator may be formed by proteolytic degradation of a one-chain molecule, and that this degradation could be blocked by the addition of the protease inhibitor, aprotinin, during purification. Human melanoma plasminogen activafor, prepared in the absence of aprotinin, consists of proteins with molecular weight of 33,000 and 39,000, while the native protein is thought to have a molecular weight of 72,000. Furthermore, the two species are closely related in peptide composition, one probably being derivated from the other (Rijken and collen, 1981). Even in the presence of aprotinin during purification, we obtained two proteins with molecular weight of ~77,000 and 35,000 proteins that both had activity (Fig. 4). However, most of the activity was recovered in the protein of ~77,000 and the specific activity of this protein seemed to be much higher than that of the 35,000 protein. This finding is consistent with results on molecular weight of the material produced by F9 cells treated with retinoic acid (Marotti et al., 1982). It is still unknown whether these two molecules are quite distinct or the 35,000 protin is a degradation product of the ~77,-000 protein. We achieved further purification by isoelectric focusing, obtaining a molecular weight of ~77,000 protein having enzyme ac-

tivity and an isoelectric point of 7.3 ± 0.1 (Fig. 5). The difference in molecuar weight of the ~77,000 protein seen in Fig. 4A and Fig. 5 might be due to a difference in carbohydrate composition or to the presence or absence of an additional peptide (Pennica et al., 1983).

Purified plasminogen activator of teratocarcinoma cells was weakly basic (Fig. 5), which seems to be a character common of tissue-type and urokinase-type plasminogen activators (Binder et al., 1979; Strickland et al., 1983). The antigenic properties of the plasminogen activator of teratocarcinoma cells induced with sodium butyrate seem to be those of the tissue-type activator (Fig. 3).

The mechanism of induction of secretion of plasminogen activator with sodium butyrate is not clear. The cells producing plasminogen activator on induction with sodium butyrate are probably parietal endoderm cells differentiated from teratocarcinoma stem cells, as on induction with retinoic acid.

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