AN ENZYMATIC FUNCTION ASSOCIATED WITH TRANSFORMATION OF FIBROBLASTS BY ONCOGENIC VIRUSES*

I. Chick Embryo Fibroblast Cultures Transformed by Avian RNA Tumor Viruses

By J. C. UNKELESS, A. TOBIA, L. OSSOWSKI,‡ J. P. QUIGLEY, D. B. RIFKIN,§ and E. REICH

(From The Rockefeller University, New York 10021)

(Received for publication 27 September 1972)

The biochemical identification of functions that are specific to malignant cells is important both for understanding the mechanism(s) of oncogenic transformation and for formulating rationally selective approaches to the chemotherapy of cancer. A variety of biochemical differences between normal and transformed cells is recorded in the literature; most of these involve small quantitative differences which, in terms of therapeutic goals, are of little significance. The unique metabolic characteristic of tumor cells, aerobic glycolysis, is also part of a biochemical continuum that includes normal cell behavior since a minority of tumors show no aerobic glycolysis and the level of aerobic glycolysis (i.e. the extent of the Pasteur effect) in the majority of tumors varies within wide limits (1).

There are a number of indications (2–4) that neoplasia may be associated with changes in hydrolytic enzymes. The most impressive of these is the observation of Fischer (5) that primary explants of viral sarcomas obtained from chickens rapidly lyse plasma clots, whereas explants of normal connective tissues do not. Under the conditions of Fischer's experiments, the observed fibrinolytic activity might have been due to contaminating cellular elements since viral sarcomas in chickens are commonly hemorrhagic and foci of necrosis and inflammation may be disseminated throughout the substance of the tumor masses. Nevertheless, this fibrinolytic activity of the tumor explants differs qualitatively from the behavior of normal tissues since the latter do not lyse plasma clots even after long periods of incubation and extensive growth. It therefore appeared desirable to establish whether Fischer's findings could be reproduced under conditions, such as those in cell culture, that exclude cellular

* Supported in part by grants from the American Cancer Society (E-478) and the National Institutes of Health (CA 08290-07) U. S. Public Health Service.

‡ Fellow of the N. Y. Heart Association.

§ Supported by ACS Faculty Research Award No. PRA-99.

contamination, autolysis, and inflammation. The experiments outlined below demonstrate that this is indeed the case: transformation of fibroblast cultures of avian or mammalian origin, by either RNA or DNA tumor viruses, is associated with the development of a greatly increased fibrinolytic activity. The expression of this activity by transformed cells requires not only factors elaborated by the cells themselves, but factors in serum as well. Furthermore, there are indications that the sera of tumor-bearing animals contain protein inhibitors of the fibrinolysin which are not detectable in the sera of normal controls. The increased fibrinolysis is probably not due to changes in amounts of lysosomal enzymes, since a comparison of these in normal and virus-transformed chick fibroblasts has revealed no significant differences.

Materials and Methods

Cell Culture and Virus Infections.—The procedures used in the preparation and maintenance of normal and transformed chicken embryo fibroblasts and in the isolation, titration, and infection by Rous sarcoma virus were exactly as described in previous reports (6). Unless otherwise specified, the virus used in most experiments with chick cells was a high titer derivative of Schmidt-Ruppin virus antigenic subgroup D (6), and all of the chick cultures were ordinarily grown in Eagle's medium supplemented with 10% fetal bovine serum (10%, vol/vol).

The additional virus strains used in some experiments were obtained as follows: RSV-(RAV-1),[1] B77, SR-RSV, RAV-50, MSV, from Dr. H. Temin; RSV(RAV-2), RAV-2, from Dr. H. Hanafusa; TS-5-SR-RSV, from Dr. G. S. Martin; influenza, Sindbis, SV5, from Dr. R. W. Compans; Sendai and Newcastle disease virus, from Dr. W. Mountcastle; vesicular stomatitis virus, from Dr. J. MacSharry; vaccinia, from Dr. S. Silverstein.

Embryonic fibroblast cultures were also prepared by trypsinization of 10-day old golden hamster embryos and 15-day old C57 black mouse embryos or rat embryos. The cultures were maintained in F10 medium (7) or in Dulbecco modified (8) Eagle's medium supplemented with 10% of fetal bovine serum. Hamster secondary cultures were infected by exposure to SV-40 virus (obtained from Dr. A. Shatkin and propagated in BSC-1 cells) at the multiplicity of 30 plaque-forming units (PFU) per cell. Clones were isolated after 12 days and scored for SV-40-T-antigen-positive nuclei by fluorescent antibody staining (9). One clone that was selected for further study formed colonies in agar, produced multilayered sheets of cells in monolayer culture, and did not release infectious SV-40 virus.

Mouse embryo secondary cultures were infected with MSV virus. The cells were treated for 1 h before infection with diethylaminoethyl (DEAE)-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) 25 μg/ml. Morphological transformation could be observed in the majority of cells 2–3 days after infection. Mouse fibroblast 3T3 cells and rat fibroblasts were

[1] *Abbreviations used in this paper:* BrTu, 5-bromotubercidin; CEF, chick embryo fibroblasts; EPGB, ethyl-*p*-guanidinobenzoate (19); FUR, 5-fluorouridine; HF, cell-free and serum-free culture supernatant fluid obtained by incubating monolayer cultures with Eagle's medium (see Materials and Methods, Part I). MSV, mouse sarcoma virus; NPGB, nitrophenyl-*p*-guanidinobenzoate (19); PBS, phosphate-buffered saline (0.01 M phosphate buffer pH 7.4, 0.15 M NaCl (26); RAV, Rous-associated virus; RSV, Rous sarcoma virus; SR-RSV, Schmidt-Ruppin strain of Rous sarcoma virus; STI, soybean trypsin inhibitor, fraction VI (29); TCA, trichloroacetic acid; TD, 0.024 M Tris pH 7.4, 0.14 M NaCl, 0.05 M KCl, 0.0037 M Na_2HPO_4 (27); TS-5-SR-RSV, temperature-sensitive mutant of SR-RSV.

infected with MSV virus in a similar way. A 3T3 mouse fibroblast cell line and derivatives that had been transformed by SV-40 virus (SV-40—3T3) were obtained from Dr. A. Goldberg.

Fibrinogen and [^{125}I]Fibrinogen.—Crude bovine fibrinogen was purchased from Pentex Biochemical, Kankakee, Ill., and was further purified by precipitation according to Laki (10). The fibrinogen thus prepared was 96% precipitable by an excess of bovine thrombin. The purified fibrinogen was iodinated according to the procedure of Helmkamp et al. (11). A threefold molar excess of ICl relative to fibrinogen was added to 2–10 mCi of carrier-free [^{125}I]Na and this was rapidly added to 10 mg of fibrinogen dissolved in a volume of 1 ml. After passage over a 1-ml column of Dowex-1-Cl^- and dialysis, 40–60% of the initial ^{125}I remained bound to the fibrinogen; this represents an average of 1.5 atoms of I incorporated per molecule of fibrinogen. On reaction with commercial bovine thrombin, 87% of the radioactivity was converted to insoluble material (fibrin) and 13% was released as small fragments, presumably fibrinopeptides.

Preparation of Petri Dishes Coated with Films of [^{125}I]Fibrin.—A preparation of [^{125}I]fibrinogen is diluted with nonradioactive carrier to a desired final specific radioactivity. Aliquots of a solution of [^{125}I]fibrinogen dissolved in phosphate-buffered saline (PBS) are pipetted into sterile Petri dishes (usually 0.1 ml); the resulting mixture is spread evenly over the surface of the plate with a glass rod and dried by incubation at 45°C for 24 h. The dishes are then incubated at 37°C overnight with regular cell culture medium, including serum. Since the serum contains an excess of thrombin, a large fraction of the fibrinogen coating is converted to fibrin during this procedure. Approximately 20% of the radioactivity is solubilized; this compares favorably with the amount released (13%) when the same preparation of fibrinogen is treated with an excess of purified bovine thrombin, and suggests that the residual protein on the plates is largely fibrin. After completion of the incubation the medium is removed and the plates washed once with 0.024 M Tris pH 7.4, 0.14 M NaCl, 0.05 M KCl, 0.0037 M Na_2HPO_4 (TD) before use. A concentration of fibrinogen of 10 $\mu g/cm^2$ was used except where noted. Radioactivity released into the supernatant was assayed using a Packard gamma spectrometer (Packard Instrument Co., Downers Grove, Ill.). When necessary, insoluble [^{125}I]fibrin on the culture dishes was digested by repeated treatment with trypsin and radioactivity assayed as usual. The range of variation in the amount of insoluble [^{125}I]fibrin that remains adherent to the surface of the Petri dishes is less than 10%.

Preparation of Harvest Fluids.—Serum-free culture fluids (HF) from normal and transformed cells were prepared after first washing the cells with TD. In the case of chick embryo cultures, the cells were then incubated in regular medium supplemented with 2.5% chicken serum for 1 h, again washed with TD, and finally incubated in Eagle's medium for 16–18 h. This serum-free fraction, designated HF, was collected and the cultures were then maintained in regular medium containing 10% of fetal bovine serum. The cultures survive this treatment effectively and can be cycled several times. Culture fluids from other cells were prepared by careful washing of the cells with TD followed by incubation in Eagle's medium for 16–18 h as for chick embryo cultures.

Macromolecule Synthesis and the Effects of Inhibitors.—The procedures used to monitor macromolecule synthesis and the effects of selected inhibitors were applied to monolayer cultures in the exponential phase of growth exactly as described previously (12). The experimental conditions are given in the individual figure legends.

Infection of Chicks with RSV and Preparation of Sera.—11-wk old SPAFAS chickens were injected in the wing web with approximately 2×10^5 focus-forming units (FFU) of RSV. After 4 wk tumors were clearly present in more than 30% of the infected birds. Normal and infected birds were then bled by cardiac puncture. After standing for 1 h at room temperature, the blood was stored for 24 h at 4°C and then centrifuged at 2000 rpm for 20 min in the PR-6 International centrifuge (International Equipment Co., Needham Heights, Mass.). The serum was filtered through Millipore filters and immediately assayed for activity.

Lysosomal Enzyme Assays.—Cells growing on 100-mm culture dishes were treated with 2 ml of trypsin (0.5 mg/ml) dissolved in TD for 6 min at room temperature. After addition of 4 ml of medium with serum the cells were collected by low-speed centrifugation, resuspended, and swollen in 2 ml of cold hypotonic saline (10 mM Tris HCl pH 7.3, 10 mM NaCl, 1 mM $MgCl_2$). The cells were centrifuged, resuspended in cold homogenizing medium (sucrose 0.25 M, ethylenediaminetetraacetate [EDTA] 1 mM, pH 7.4), and broken with 10–20 strokes in a Dounce homogenizer with a tight-fitting pestle. Cell breakage was judged to be better than 90% by phase-contrast microscopy. The homogenate was centrifuged at 500 *g* for 10 min and the supernatant was set aside. The same volume of homogenizing medium used previously (1 or 2 ml) was added and nuclei and unbroken cells were resuspended with several strokes in the homogenizer. After centrifugation the two supernatants were combined.

All assays for lysosomal enzymes in the cell homogenate thus obtained were performed at 37°C for 1 h in the presence of 0.1% Triton X-100 to release any bound enzymes. Final incubation volume was 0.2 ml and 0.05–0.1 ml of the extract was assayed. All assays were performed in triplicate with appropriate standards and blanks.

Acid phosphatase (E.C.3.1.3.2) was assayed according to Appelmans, Wattiaux, and de Duve (13) using β-glycerophosphate as the substrate. The protein was precipitated with 0.3 ml of 13% trichloroacetic acid (TCA) and after centrifugation aliquots of the supernatant were assayed for released orthophosphate (14).

β-*N*-acetylglucosaminidase (E.C. 3.2.1.30) and β-galactosidase (E.C. 3.2.1.23) were assayed according to Sellinger et al. (15) using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and *p*-nitrophenyl-β-D-galactoside, respectively. Protein was precipitated as before and the *p*-nitrophenol content was measured at 410 nm after addition of 0.75 ml of buffer (0.25 M glycine·Na_2CO_3 pH 10, containing 0.2 M NaOH) to 0.25 ml of the supernatant.

Cathepsin A was assayed according to Iodice, Leong, and Weinstock (16) using 20 mM carbobenzoxy-L-glutamyl-L-tyrosine as substrate. Reactions were terminated by addition of 0.02 ml 50% TCA. After centrifugation a 0.15 ml aliquot of the supernatant was assayed by the ninhydrin method (17). The ninhydrin blanks due to substrate alone and protein without substrate were determined and appropriate corrections were made.

Protein was determined by the method of Lowry *et al.* (18) using bovine serum albumin as the standard.

Assay of Fibrinolysis by Cells and by Cell-Free Preparations.—When fibrinolytic activity is assayed in cell cultures, the cells are plated onto Petri dishes coated with [^{125}I]fibrin in media supplemented with fetal bovine serum to minimize fibrinolysis. After attaching to the coated surface the cells are permitted to grow for the desired time, and monitoring of fibrinolysis is ordinarily initiated by changing to fresh medium; the release of radioactivity into growth medium is followed by assays either of aliquots or of the total volume of medium at selected time intervals. The range of variation in release of radioactivity by replicate cultures is 20–25%; thus, in experiments with cell cultures the average of two replicate cultures is used for each determination.

For assays of cell-free fibrinolysis, the desired reaction mixture is also incubated in Petri dishes coated with [^{125}I]fibrin. Since the range of variation, approximately 10%, is smaller than that found with cells, each determination is based on a single plate unless indicated otherwise.

In assays of fibrinolysis by cell cultures the cells are always plated onto fibrin-coated dishes in media containing fetal bovine serum because fetal bovine serum minimizes fibrinolysis. Thus, after 24 h of growth and at the cell densities used in this type of experiment, less than 10% of the fibrin is removed from the surface of the dish.

Sera were purchased from Miles Laboratories Inc. (Elkhart, Ind.) and Grand Island Biological Co. (Grand Island, N. Y.), and powdered culture medium was purchased from GIBCO. All Petri dishes were disposable Falconware. Doctors H. Wood and R. Engle, Drug Develop-

ment Branch, National Cancer Institute, generously provided soybean trypsin inhibitor, 5-bromotubercidin (BrTu) (synthesized by Dr. L. Townsend, University of Utah), and nitrophenyl-*p*-guanidobenzoate (NPGB); samples of NPGB and ethyl-*p*-guanidobenzoate (EPGB) (19) were also generously made available by Dr. Elliott Shaw, Brookhaven National Laboratory. All other materials and reagents were of the best commercially available grades. Tritium-labeled metabolites and precursors were purchased from Schwarz Bio Research Inc. (Orangeburg, N. Y.) and measurements of incorporated radioactivity were made with a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Fibrinolysin in Transformed Cultures.—When fibrinogen films are deposited on the surface of Petri dishes and then incubated with regular cell culture media supplemented with serum, the fibrinogen is converted to insoluble fibrin, which is visible as wavy threads under phase-contrast microscopy. As seen from the photomicrograph in Fig. 1 *a*, normal chick embryo fibroblasts (CEF) grow on top of and between the fibrin layers; in transformed cultures, by contrast (Fig. 1 *b*), the fibrin has disappeared after 48 h of incubation. This result, therefore, provides a confirmation of Fischer's original observations (5).

A quantitative counterpart of this observation can be obtained by using plates coated with films consisting of [^{125}I]fibrin. Under these conditions, the

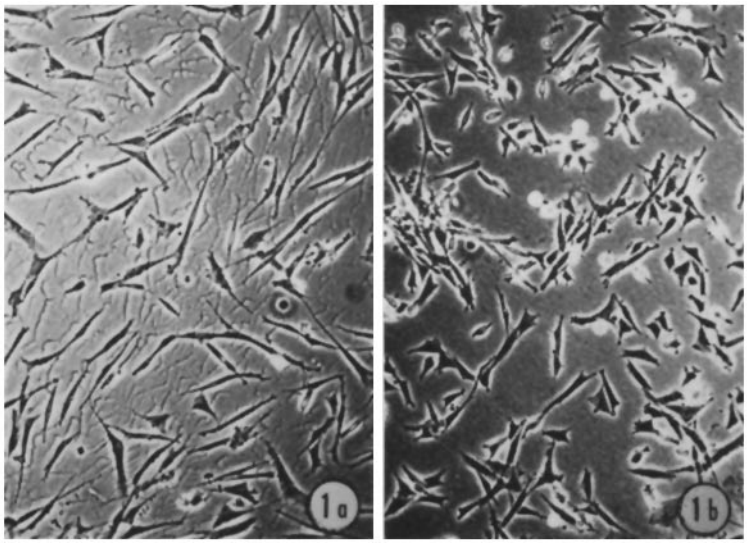

FIG. 1. Normal and RSV-transformed CEF: growth on fibrin-coated plates. Normal and RSV-transformed CEF were plated at densities of 2 and 2.2 $\times$ 10^5 cells per dish, respectively, in Petri dishes coated with fibrin (20 μg/cm^2). The medium, which was supplemented with 10% of fetal bovine serum, was changed after 16 h, and the cultures were photographed 32 h later. (1 *a*) Normal CEF: fibrin visible as wavy lines; (1 *b*) RSV-transformed culture: fibrin no longer visible. $\times$ 88.

radioactivity in the transformed cultures is released into the supernatant from the surface of the culture dish; the corresponding release in normal cultures is less than 5% of that in the transformed cells, after correction for the background given by the controls without cells. As seen in Table I the fraction of fibrin solubilized does not decrease as the fibrin concentration is increased, showing that the system has not reached saturation. Release of radioactivity by normal cells barely exceeds that found in control dishes that contain growth medium but no cells.

Degradation Products from [^{125}I]Fibrin.—The nature of the radioactive materials released into the medium by cultures of transformed cells was examined by gel permeation chromatography on Sephadex G-100 (Fig. 2). The starting

TABLE I

Fibrinolytic Activity of Normal and RSV-Infected Chick Embryo Fibroblasts

Fibrin	Radioactivity released into growth medium (percent of total)		
	RSV-transformed CEF	Normal CEF	No cells
μg *per* cm^2	%	%	%
1	8.5	NT*	3
2.5	26	6	5
5	26	7	5
15	28	6	5
34	21	7	5

Varying amounts of [^{125}I]fibrin of constant specific radioactivity (1.8×10^4 cpm/μg) were coated onto 60-mm Petri dishes as described in Methods. After incubation with medium for 24 h the dishes were washed and inoculated with transformed and normal CEF at 2.2×10^5 cells and 2×10^5 cells per plate, respectively. Medium was removed and assayed for radioactivity 24 h later.

* Not tested.

material, [^{125}I]fibrinogen, is completely excluded from the gel and emerges at the void volume of the column (Fig. 2 A). This is in contrast to the material found in the supernatants from transformed cultures, in which 84% of the radioactivity is included in the gel and consists of peptide fragments of heterogeneous size (Fig. 2 B). When tested for solubility in the presence of trichloroacetic acid (5% final concentration wt/vol), 25% of the radioactivity was acid soluble The fibrinopeptides, formed by the action of thrombin on [^{125}I]fibrinogen, are by comparison 13% acid soluble. Thus the [^{125}I]fibrin is substantially degraded, and a large fraction of the resulting fragments is acid insoluble. This implies that the enzyme(s) responsible for fibrinolysis has restricted specificity for either peptide sequence, size, conformation, or a combination of these.

Comparison of Transforming, Nontransforming, Cytocidal, and Temperature-Sensitive Transforming Viruses.—The difference in fibrinolytic activity between normal and transformed cultures is substantial and qualitative. Nevertheless,

it appeared desirable to perform additional experiments for testing the correlations, if any, in the appearance and activity of the enzyme with the presence and onset of cell transformation. For this purpose chick embryo fibroblast (CEF) cultures were infected with (*a*) a variety of transforming and nontransforming

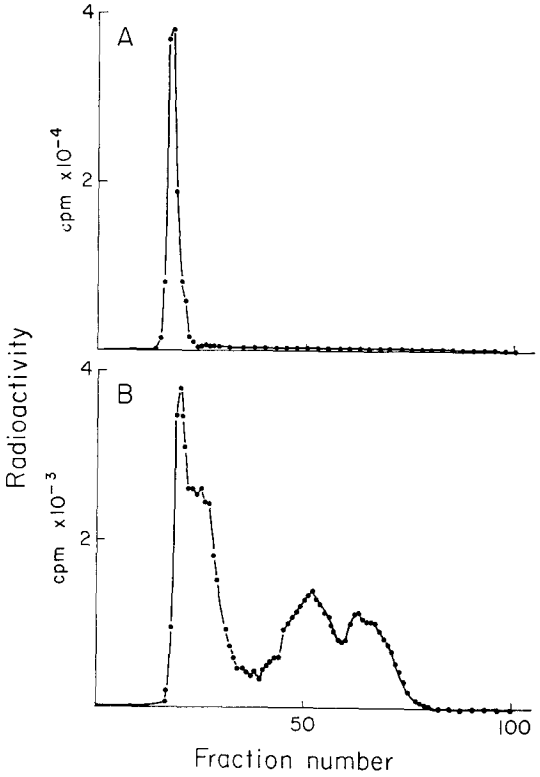

FIG. 2. (A) 1 ml, 400 μg of [^{125}I]fibrinogen (total radioactivity 1.6 $\times$ 10^5 cpm) was fractionated on the same column as described in B. (B) A 60 mm Petri dish coated with [^{125}I]fibrin (10 μg/cm^2; total radioactivity 10^6 cpm) was incubated with 2.5 ml of HF from RSV-transformed chick cells supplemented with 2.5% of chicken serum. After incubation at 37°C for 5 h, 1 ml of supernatant, containing total radioactivity of 72,000 cpm was applied to a column (41 $\times$ 2 cm) of G-100 Sephadex previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.1. The flow rate was 15 ml/h and 2-ml fractions were collected.

avian sarcoma and leukosis viruses, (*b*) a series of cytolytic viruses, and (*c*) a temperature-sensitive mutant of RSV; the latter is a strain that maintains virus production at both low (permissive) and high (nonpermissive) temperatures, but causes cell transformation only at low temperatures (20). The results of these experiments are presented in Table II and Fig. 3.

The data in Table II compares the effects of the transforming viruses with

those of nontransforming viruses. The transforming viruses include representatives from all of the antigenic subgroups (A–D), and cultures infected with any one of these viruses develop the same kind of fibrinolytic activity observed with our standard strain of Schmidt-Ruppin virus (subgroup D). Conversely, cultures infected by the nontransforming viruses fail to produce fibrinolytic

TABLE II

Fibrinolytic Activity of Chick Embryo Fibroblasts Infected with Various Viruses

Infecting virus	Radioactivity released into growth medium (percent of total)			
	5 h		17 h	
	Serum supplement			
	Fetal bovine serum	Chicken serum	Fetal bovine serum	Chicken serum
	%	%	%	%
Noninfected cells	0.5	1.3	1.4	8.1
RSV-(RAV-1)	24.4	64.8	50.4	90.6
RSV-(RAV-2)	23.0	76.0	50.0	101.8
B77	16.2	69.5	36.0	95.1
SR-RSV	16.2	73.5	36.2	101.9
RAV-2	0.2	2.0	1.5	9.5
RAV-50	0.4	1.8	1.4	9.0
Sendai	0.9		1.0	
VSV	0.8		1.1	
NDV	0.8		1.2	
Sindbis	0.8		1.3	
Vaccinia	0.8		1.2	
Influenza	2.0		5.4	
SV_5	1.4		2.1	
SV_5*	0.5		2.6	

Petri dishes (60 mm diameter) contained 10 μg[^{125}I]fibrin per cm^2 with total radioactivity of 2.5×10^4 cpm and were prepared as described in Methods. Cells were infected with leukosis viruses at least 8 days, and with cytocidal viruses 1 h before plating. The cells were plated at 10^6 cells per dish and the medium was removed for radioactivity measurements at 5 or 17 h. The concentration of fetal bovine serum was 10% (vol/vol) and the concentration of chicken serum was 2.5% (vol/vol).

* Tested 1 wk after cells were infected.

activity. These data indicate that the development of the fibrinolytic activity is closely correlated with cellular transformation.

The behavior of the cytolytic viruses is likewise consistent with the preceding conclusion. As also seen in Table II, cultures were infected with various cytocidal strains, including representatives of myxo-, paramyxo-, and arborviruses, as well as the DNA poxvirus vaccinia. These cultures produce very little fibrinolytic acitivity during the course of the infection, which culminates in cell destruction and lysis. This result shows that the formation of the fibrinolysin is

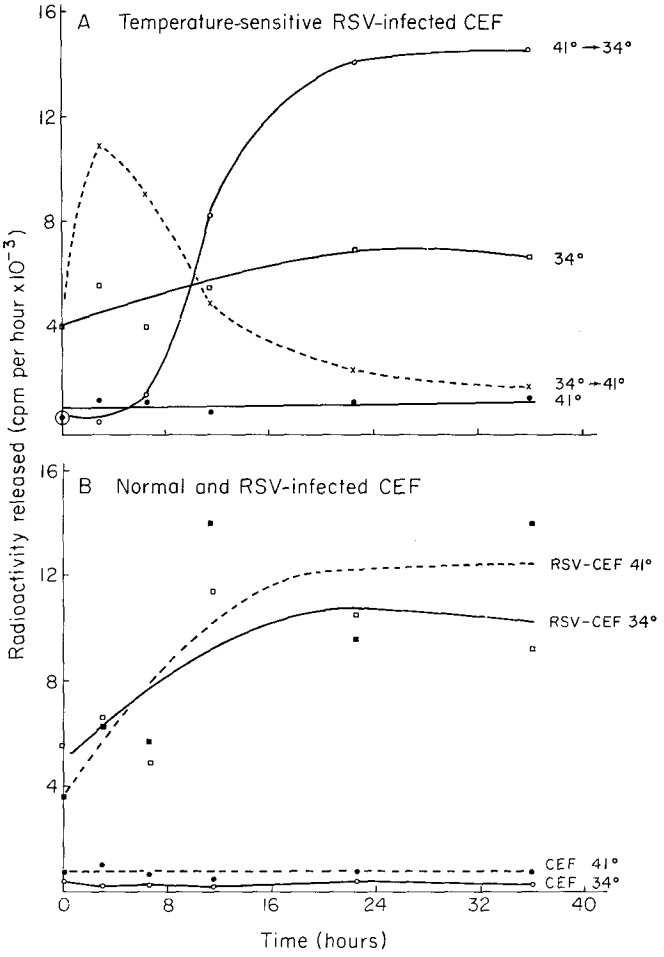

FIG. 3. Fibrinolytic activity of normal CEF and of CEF infected with SR-RSV or TS-5-SR-RSV: effect of temperature shifts. Normal CEF and CEF infected by standard (SR-RSV) (B); temperature-sensitive Rous sarcoma virus (TS-5-SR-RSV) (A) were grown at 41°C and plated on 35-mm dishes containing [^{125}I]fibrin (10 μg/cm^2; 3 $\times$ 10^6 cpm). The cell density was 4.9 $\times$ 10^4 cells per dish for normal cells and 5.7 $\times$ 10^4 cells per dish for both infected cultures. Some of the cultures from each group were incubated at 34°C and others at 41°C. The medium was changed after 24 h, and the cells were permitted to grow for a further 24 h period, at which time the medium was removed and assayed for radioactivity (0 time). Some of the cultures infected with TS-5-SR-RSV were then transferred from 34° to 41°C, and vice versa. Thereafter the medium from cell cultures was exchanged and assayed for radioactivity at the indicated times. The cultures grown at 41°C after plating had a higher cell density than those maintained at 34°C, and the results have not been normalized with respect to cell number. The increased rate of fibrinolysis that follows the transfer of temperature-sensitive cultures from 34° to 41°C is due to an increase in the rate of the enzyme that is present in the medium at the time of the temperature shift.

not merely associated nonspecifically with virus infection and/or cell lysis. It is particularly noteworthy that the "temperate" paramyxovirus SV-5 does not give rise to fibrinolytic activity.

Strong support for an intimate correlation between cellular transformation and the appearance of fibrinolysin is given by the behavior of cultures infected with an RSV mutant whose ability to cause transformation is temperature sensitive. Such cultures do not show a significant level of fibrinolysin at the high, nonpermissive temperature that prevents transformation (Fig. 3 A). This is in contrast to the behavior of cultures transformed by the standard strain of Schmidt-Ruppin virus, in which fibrinolytic activity is expressed at 34°C and 41°C (Fig. 3 B); no fibrinolysis is produced at either temperature by normal CEF (Fig. 3 B). If the appearance of enzyme were related to the process of transformation, activity should be detectable shortly after the cultures are shifted to the low, permissive temperature; this is, in fact, the case, and the time course of enzyme appearance, approximately 8–10 h, is shorter than that reported for morphological changes under the same conditions, namely 12–18 h (20). The converse experiment, in which cultures are transferred from the low to the higher, nonpermissive temperature, shows that activity does not increase after the shift and declines over a period of hours. This finding is important for two reasons: (*a*) it is consistent with the expectation, derived from the preceding experiment, that the formation of enzyme should not continue at nonpermissive temperatures, and (*b*) it indicates that the enzyme, once formed, is measurably stable during an 8 h period and is not rapidly metabolized or inactivated. This is reassuring because it suggests that the failure to detect enzyme in cultures infected by cytocidal, or by nontransforming viruses is probably not due to extremely rapid turnover or inactivation; moreover, this slow rate of decay of enzyme activity is an indication that the factors responsible for fibrinolysis are not themselves thermolabile.

Appearance of Fibrinolytic Activity, Its Relationship to Cellular Macromolecule Synthesis.—The time lag that intervenes between the shift of cultures to permissive temperatures and the initial appearance of enzyme provides the opportunity for testing the relationship between the induction of fibrinolytic activity and cellular macromolecule synthesis. Accordingly, cultures infected with the temperature-sensitive mutant were treated with suitable inhibitors, then transferred to the lower permissive temperature and monitored for enzyme action. As seen in Fig. 4, cytosine arabinoside, which rapidly and completely blocks DNA synthesis in fibroblast cultures, does not prevent the anticipated appearance of fibrinolysin. However pretreatment of cultures with adequate concentrations of either actinomycin or cycloheximide (Fig. 5) blocks enzyme formation at the permissive temperature, suggesting that both new RNA and protein synthesis are required for enzyme production under these conditions. The independence of any relationship to DNA synthesis, and the need for protein syn-

thesis to precede enzyme formation both correlate well with the temperature-sensitive morphological change, whose requirements are identical (20, 20 *a*).

The requirement for RNA synthesis to precede fibrinolysin biosynthesis can be examined in more detail by means of the nucleoside inhibitors 5-fluorouridine

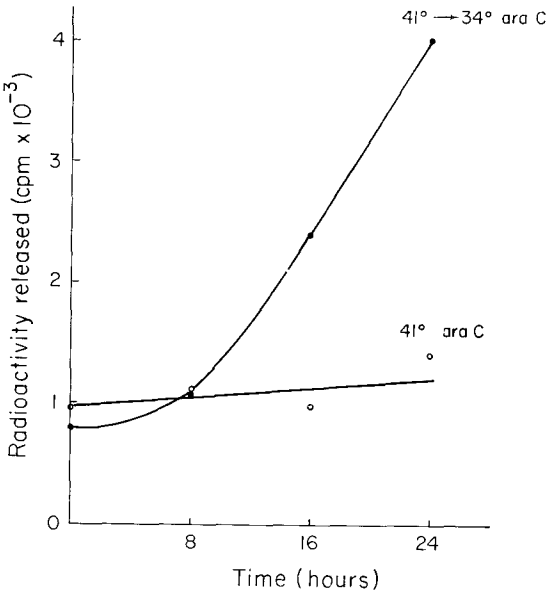

FIG. 4. Fibrinolytic activity of CEF infected with TS-5-SR-RSV: effect of treatment with cytosine arabinoside. Chick embryo fibroblasts, infected with TS-5-SR-RSV and grown at 41°C, were plated on 35-mm Petri dishes at a density of 1.5×10^5 cells per dish. The dishes were coated with [^{125}I]fibrin containing a total of 2×10^6 cpm and the cultures were placed at 41°C after plating. The medium was removed and replaced by fresh medium after 20 h of incubation. Cytosine arabinoside (ara C) (final concentration 0.3 mM) was added to the cultures 27½ h after plating; this concentration was then maintained throughout the duration of the experiment. After 30 min of incubation at 41°C in the presence of cytosine arabinoside the medium was exchanged for fresh medium of identical composition (0 time); one pair of cultures was transferred to 34°C, another pair remaining at 41°C and serving as control. The medium was changed and assayed for radioactivity at the indicated times. ●——● 41° → 34°C; ○——○ 41°C.

(FUR) and 5-bromotubercidin (BrTu). It is known from previous work (21) that FUR irreversibly inhibits ribosomal RNA synthesis but does not interfere either with mRNA synthesis or mRNA-dependent processes such as in vitro differentiation of skeletal muscle (22) or the multiplication of RSV.[2] In contrast to FUR, BrTu reversibly inhibits both mRNA- and rRNA-directed functions

[2] Brdar, B., D. B. Rifkin, and E. Reich. 1972. Studies of Rous sarcoma virus; effects of nucleoside analogues on virus synthesis. *J. Biol. Chem.* In press.

such as myogenesis and RSV production. From the data in Fig. 6 it is apparent that a pretreatment with FUR (sufficient to block rRNA synthesis completely) does not prevent fibrinolysin formation after exposure to permissive temperatures; this result is consistent with the expectation that only mRNA synthesis should be needed for enzyme synthesis. The effect of BrTu is also in accord with this finding: when cultures are exposed to BrTu before the temperature shift, no enzyme is detected after transfer to the permissive temperature; how-

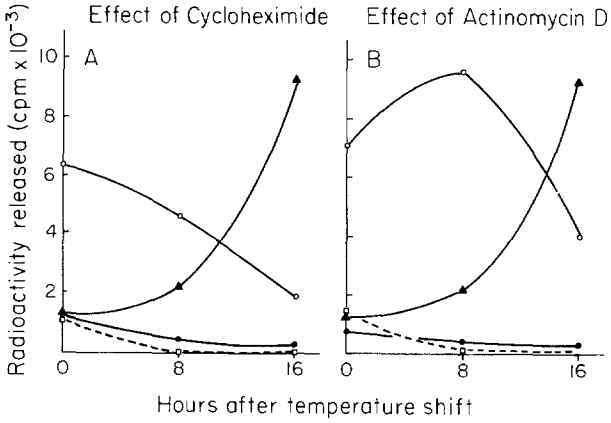

FIG. 5. Fibrinolytic activity of CEF infected with TS-5-SR-RSV: effect of treatment with cycloheximide or actinomycin. Chick embryo fibroblasts, infected with TS-5-SR-RSV and grown at 41°C, were plated on 35-mm dishes at a density of 6×10^4 cells per dish. The dishes were coated with [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2.2×10^5 cpm). After plating the cultures were incubated at either 34°C or 41°C and the medium changed at 8-hourly intervals. At 16 h after plating the medium was removed for radioactivity measurement (0 time), fresh medium containing the inhibitors was added, and some of the cultures were transferred from 41° to 34°C, others remaining at 41°C and serving as controls. The medium in all cultures was exchanged and radioactivity assays performed at 8-hourly intervals, and the concentrations of inhibitors was maintained in the relevant cultures for the duration of the experiment. Cycloheximide and actinomycin D, where present, were at 2 μg/ml and 1 μg/ml, respectively. ○——○ 34°C with inhibitors; ●——● 41°C with inhibitors; △——△ 41° → 34°C control; □——□ 41° → 34°C with inhibitors.

ever, the subsequent removal of BrTu allows enzyme formation at the permissive temperature to proceed (Fig. 7). The time lag in enzyme appearance that is observed after removal of BrTu is similar to that which occurs after a simple downward temperature shift. The effects of FUR and BrTu therefore both reinforce the conclusion that mRNA synthesis must precede expression of fibrinolytic activity under these conditions.

Localization of the Fibrinolysin in Growing Cultures.—After all the preceding observations had been suitably documented, numerous and uniformly unsuccessful attempts were made to demonstrate the presence of enzyme in the cell-

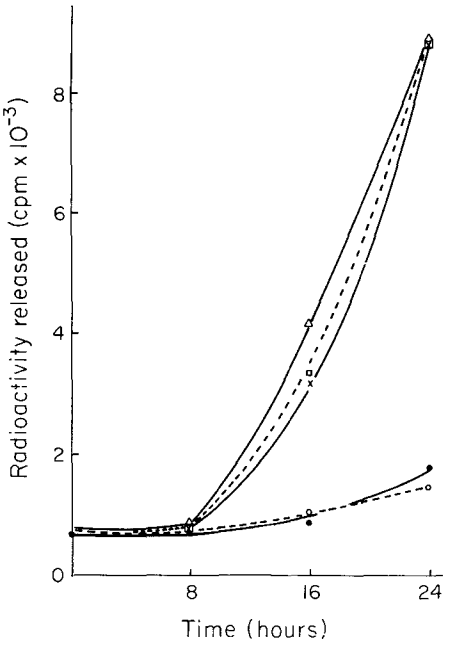

FIG. 6. Fibrinolytic activity of CEF infected with TS-5-SR-RSV: effect of treatment with 5-fluorouridine. Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2 $\times$ 10^6 cpm) were prepared as described in Methods. Each dish received 1.5 $\times$ 10^5 CEF previously infected with TS-5-SR-RSV and maintained at 41°C. After plating the cultures were incubated further at 41°C and the medium was changed at 20 h; FUR (10 μg/ml) was added to different cultures at 20, 24, or 27½ h after plating, respectively, and the concentration of FUR was maintained for the duration of the experiment. At 28 h after plating, the medium was removed from all cultures and assayed for radioactivity (0 time). After receiving fresh medium the cultures were transferred to 34°C, and aliquots of the medium were removed for radioactivity determinations every 8 h. The FUR pretreatment does not inhibit the onset of fibrinolysis after the temperature shift. ×——× 41° → 34°C, FUR added at 27½ h; □——□ 41° → 34°C, FUR added at 24 h; △——△ 41° → 34°C, FUR added at 20 h; ●——● 41°C, FUR added at 27½ h; ○——○ 41°C, FUR added at 24 h.

free supernatants. This suggested that the fibrinolysin was cell bound, probably to the cell surface. To test for this normal and transformed cell monolayers were placed in contact with cover slips that had been coated with a film of [^{125}I]-fibrin only on one side. The presence of enzyme in the culture medium, even if short-lived, should produce fibrinolysis even if the cover slip were placed in the culture with the coated surface facing upwards and away from the cells. As seen in Table III, fibrinolysis occurs only when the coated surface of the cover slip is downwards and in contact with the underlying transformed cell monolayer; no fibrinolysis is observed with either orientation of the cover slip in

normal CEF cultures. These results imply that the fibrinolytic activity is cell associated; however, this behavior appears to hold only under certain conditions, and it is dependent on the nature of the serum present in the growth medium (*vide infra*).

Effect of Different Sera.—Because different sera vary in their ability to permit

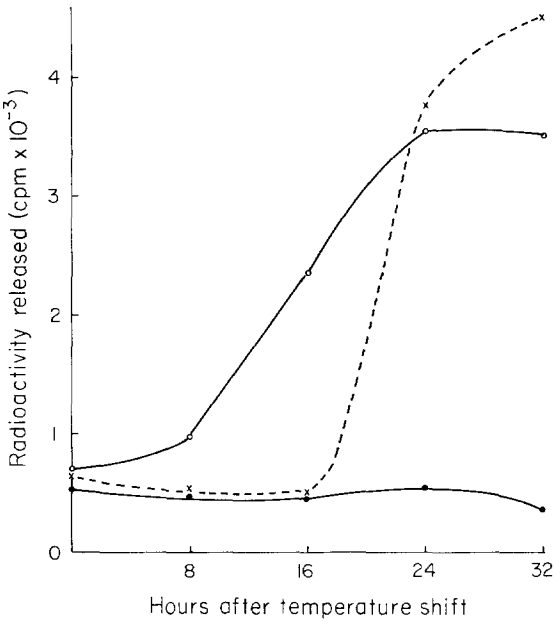

FIG. 7. Fibrinolytic activity of CEF infected with TS-5-SR-RSV: effect of treatment with bromotubercidin. Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2, total radioactivity 2 $\times$ 10^6 cpm) were prepared as described in Methods. Each dish was inoculated with 1.5 $\times$ 10^5 cells previously infected with TS-5-SR-RSV and grown at 41°C, and the plates were maintained at 41°C. The medium was changed at 20 h after plating and BrTu (10 μg/ml) was added to all dishes 7½ h later. After 30 min of incubation with BrTu the medium was removed for determination of radioactivity (0 time) and the dishes were transferred from 41° to 34°C. The medium in some of the cultures was exchanged for fresh medium without BrTu either at the time of or 8 h after the temperature shift. (O——O) BrTu removed at zero time; (×-----×) BrTu removed 8 h after temperature shift; (●——●) BrTu present continuously.

the expression of some parameters of transformation (such as morphological changes, growth in or under soft agar overlay, etc.), it appeared likely that corresponding differences might be found with respect to the fibrinolytic activity of transformed cultures. A large number of sera were accordingly tested in place of the standard fetal bovine serum used in our growth media. The behavior of a representative selection of sera is given in Table IV. It is apparent that fibrinolytic activity is markedly influenced by the nature of the serum. Of the

TABLE III

Fibrinolysis by Cells in Contact with [^{125}I]Fibrin-Coated Glass Cover Slips

	Radioactivity released into medium (percent of total)	
	Fibrin layer face down	Fibrin layer face up
	%	%
Control (no cells)	0.20	0.27
Normal CEF	0.27	0.31
RSV-infected CEF	3.15	0.38

Glass cover slips (12 mm diameter) were coated on one side with [^{125}I]fibrin (10 μg/cm^2) containing a total radioactivity of 8.0×10^4 cpm. After incubation in serum-supplemented growth medium for 24 h, they were carefully washed and placed fibrin side either up or down in 60-mm dishes containing 2×10^5 normal or 2.2×10^5 RSV-transformed CEF, respectively. The medium was changed at daily intervals and assayed for radioactivity. The values given correspond to the total radioactivity released into the medium between 24 and 48 h after plating.

TABLE IV

Fibrinolytic Activity of RSV-Transformed Chick Embryo Fibroblasts: Effects of Different Sera

Test sera Serum concentration (vol/vol):	Radioactivity released into growth medium (percent of total)	
	1%	10%
	%	%
Fetal bovine	9.5	8.5
Bovine	14	10
Turkey	11	8.5
Chicken	41	73
African green monkey	40	52
Dog	11	6
Guinea pig	10	8

Petri dishes (60 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 10^6 cpm per dish) were prepared as described in Methods. RSV-infected CEF were plated in 10% fetal bovine serum at 2.2×10^5 cells per dish. The cells were washed with TD 24 h after plating and changed to medium with the different sera. The medium was removed and assayed for radioactivity after 16 h.

samples used in this experiment, chicken and monkey sera are clearly the most effective for promoting fibrinolysis; indeed chicken serum is even more active than suggested by these data since the observed degree of fibrinolysis corresponds to the degradation of most of the available fibrin in the Petri dish. (However, the difference between normal and transformed cultures is maintained even with chicken serum [Table II].) The rate of fibrinolysis depends to some extent on serum concentration, as shown by the higher activity observed when the chicken serum level is raised from 1% to 10%.

One finding of interest is given by mixtures of sera that individually yield

different levels of fibrinolysis. This is shown by the effect of adding progressively increasing amounts of fetal bovine serum (a relatively "nonactivating" serum) to a basic fixed level of chicken serum. The result (Table V) indicates that increasing amounts of fetal bovine serum depress the level of fibrinolysis below that found with chicken serum alone. Thus fetal bovine serum must contain an inhibitor of the fibrinolytic system, and it can be assumed that the failure of this serum to mediate the same level of activity as chicken or monkey sera reflects the presence of the inhibitor. This observation provides another instance of parallelism between fibrinolysis and other manifestations of transformation since it has been reported that fetal bovine serum inhibits focus formation by RSV under agar (23). The behavior of fetal bovine serum is not unique, since

TABLE V

Inhibition of Fibrinolytic Activity of RSV-Infected CEF by Fetal Bovine Serum

Percent serum (vol/vol)		Radioactivity released into medium (percent of total)	
Chicken	Fetal bovine	8 h	24 h
		%	%
5	0	12.5	100
5	1	6	58
5	5	1.7	19
0	5	1.2	9

Petri dishes (60 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 10^6 cpm) were prepared as described in Methods. The dishes were then inoculated with RSV-infected CEF (2.2×10^5 cells per dish), incubated for 24 h in media containing 10% of fetal bovine serum, and then washed with buffered saline. Two dishes were reserved and used to determine the [^{125}I]fibrin remaining on the surface at this time (100%). The remainder were incubated in media containing one of the indicated serum supplements. The medium was sampled for radioactivity at the indicated times. The fraction of radioactivity released is based on the total remaining at the time that the medium was changed.

mixtures of other "activating" and "nonactivating" sera give identical results, although the proportions of inhibitory serum required to produce a given degree of enzyme inhibition vary according to the individual mixture.

Assay for Cell-Free Fibrinolysin: Demonstration of Cell Factor and Serum Factor.—The high level of fibrinolytic activity obtained with the activating sera in transformed cultures encouraged further efforts to detect the responsible enzyme under cell-free conditions. This could be achieved by using growth media supplemented with chicken serum; the cell-free supernatant fluids from such transformed cultures contain fibrinolytic activity as shown by the ability to digest fibrin films. However the enzymatic activity obtained in this way was difficult to fractionate.

It appeared likely that the fibrinolytic activity could be generated by interaction between a serum protein and the transformed cells. To test for this,

growing monolayers of transformed cells were washed to remove the fetal bovine serum and then incubated for 1–2 h with Eagle's medium supplemented with chicken serum (2.5%). At the end of this period the medium was aspirated and discarded, and the monolayers were again washed thoroughly with buffered saline and finally incubated overnight with Eagle's medium containing no serum. The resulting fluids were collected (hereafter designated as HF) and tested for fibrinolytic activity in the presence and absence of serum; the assay was based on the release of [^{125}I]fibrin from Petri dishes coated with radioactive films. The results are presented in Table VI and reveal the following. (*a*) When

TABLE VI

Fibrinolytic Activity of HF from RSV-Infected and Normal Chick Embryo Fibroblasts: Serum Specificity

Test fluids	Serum supplement in medium	Radioactivity released into solution (percent of total)
		%
Medium	None	1.6
Medium	Chicken	1.1
Normal cell HF	Chicken	1.2
RSV-transformed cell HF	None	2.5
" " " "	Chicken	45
" " " "	Fetal bovine	6.5
" " " "	African green monkey	11
" " " "	Dog	3.7
" " " "	Hamster	2.6
" " " "	Fetal pig	1.7

Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2.5×10^4 cpm) were prepared as described in Methods. Serum-free Eagle's medium or HF (1 ml) was mixed with the indicated serum supplement to yield a final serum concentration of 2.5% (vol/vol), added to the [^{125}I]fibrin-coated plates, and incubated at 37°C for 3 h. Aliquots of the incubation medium were removed for radioactivity assays. The control value for chicken serum medium incubated in the absence of HF is the only one presented in the table. The controls for all other sera without HF gave values identical with that found with the chicken serum control.

transformed culture HF is mixed with serum, active fibrinolysis results. (*b*) Neither transformed HF, nor normal HF, nor growth medium and serum alone give fibrinolytic activity. (*c*) A mixture of normal culture HF and serum gives no fibrinolysis.

The pattern of activating and nonactivating sera found with the fibrinolytic activity of whole cultures is fully reproduced by the mixture of cell-free HF and sera; this is also shown in Table VI. Thus the transformed cells and activating sera each contain factors needed for the production of fibrinolysis; the enzymatic activity is not formed by either alone but requires the interaction of both the cell and serum factors.

As already noted above (Table IV) the rate of fibrinolysis in cultures is influenced by the concentration of activating serum. This effect is retained in the cell-free assay (Table VII). Likewise, the results of a titration given in Fig. 8 show that the rate of enzyme action at constant serum concentration also reflects the amount of HF in the reaction mixture. These findings provide the quantitative basis for purifying and characterizing both HF (cell) and serum factors. Data to be presented elsewhere show that both the cell factor and the serum factor are heat-labile proteins, and that the fibrinolysin does not require

TABLE VII

Fibrinolysis by HF from RSV-Transformed CEF: Effect of Serum Concentration

HF	Chicken serum	Radioactivity released (percent of total)
	%	%
−	0	1.5
−	0.5	1.6
−	2.5	1.7
−	10.0	2.2
+	0	2.0
+	0.1	2.9
+	0.2	3.9
+	0.5	8.5
+	1.0	13.0
+	2.5	23.0
+	5.0	31.0
+	10.0	31.0

Plates (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2.5 × 10^4 cpm) were prepared as described in Methods. Chicken serum was added to HF or medium without serum (final volume 1 ml) and incubated at 37°C for 1 h, after which the sample was assayed for radioactivity.

any small molecules or other cofactors from serum or medium. By using the digestion of [^{125}I]fibrin films as an assay, we have purified each of these factors to apparent homogeneity; full details of the isolation and purification procedures and of the enzymological characteristics of the reaction will be published separately.[3]

Inhibitors of Fibrinolysis.—Preceding experiments (Table V) indicate that fetal bovine serum contains an inhibitor of the fibrinolytic activity when the latter is measured in growing cultures of transformed cells. The data in Table VIII show that this inhibition is also observed under cell-free conditions when HF is mixed with medium that contains serum. Under these conditions stepwise increments of fetal bovine serum progressively inhibit the fibrinolytic

[3] Manuscript in preparation.

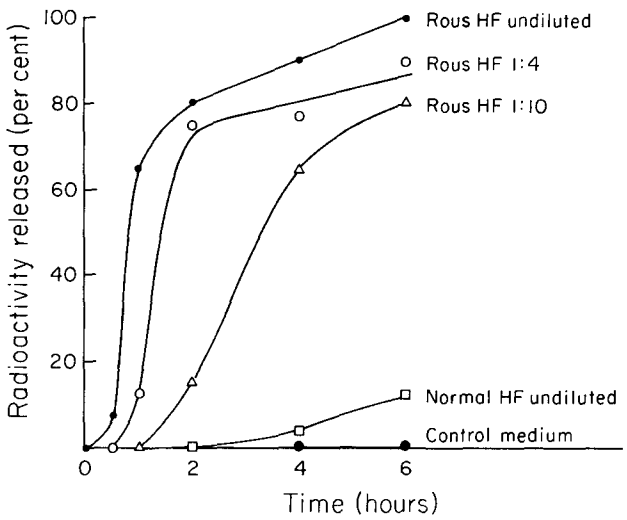

FIG. 8. Fibrinolytic activity as a function of concentration of HF. Confluent cultures of normal and RSV-transformed chick embryo fibroblasts were incubated with Eagle's medium to prepare HF (Methods). The HF obtained in this way was diluted with fresh Eagle's medium as indicated, chicken serum was added to a final concentration of 2.5%, and 1 ml of the mixture was incubated in Petri dishes (35 mm diameter) coated with [^{125}I]fibrin (10 μg/cm^2; total radioactivity 6 $\times$ 10^4 cpm). Incubation was at 37°C. At the indicated times medium was removed and assayed for radioactivity. Each value is the average of two separate determinations from duplicate assays.

TABLE VIII

Fibrinolytic Activity of HF: Inhibition by Fetal Bovine Serum

Concentration of chicken serum (percent vol/vol)	Concentration of fetal bovine serum (percent vol/vol)	Radioactivity released into solution (percent of total)	Percent of control
		%	%
2.5	0	33	100
2.5	0.5	26	79
2.5	1.5	20	60
2.5	2.5	12	36
0	2.5	6.5	20

Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2.5 $\times$ 10^4 cpm) were prepared as described in Methods. Serum supplements were added to HF from RSV-transformed CEF cultures (1 ml), and the dishes incubated for 3 h at 37°C. The radioactivity released into the solution was assayed.

reaction. Since the effect of fetal bovine serum in a cell-free system reproduces the pattern of inhibition found with the intact cellular system, the inhibitor is probably not directed against either the transformed cells or the synthesis of the cell factor, but rather against one of the components of the in vitro reaction.

We have observed that other molecules also inhibit fibrinolysis both in cultures of transformed cells and under cell-free conditions such as those used for the preceding experiments; in all cases (Table IX), the results with HF are qualitatively similar to those obtained with cell cultures. Certain preparations

TABLE IX

Fibrinolysis by HF: Effect of Various Inhibitors

Inhibitors	Inhibitor concentration	Radioactivity released into solution (percent of total)	Percent inhibition
	μg/ml	%	%
Complete system	—	38	0
Medium with chicken serum (no HF)	—	1.7	—
Complete system + nitrophenyl-*p*-guanidinobenzoate	100	1.7	100
	20	1.7	100
	5	1.7	100
Protamine sulfate*	500	7	86
	50	29	25
	5	37	3
Soybean trypsin inhibitor fraction VI	100	2.2	98
	20	6.3	88
	5	13	70
ε-Aminocaproate*	500	2.1	99
	50	16.5	60
	5	32	17
NaCl	0.5 M	2.4	98
	0.1 M	34	15
N-α-tosyl-L-lysyl chloromethyl ketone*	500	38	0
2-Nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate*	400	38	0
N-α-tosyl-L-phenylalanyl chloromethyl ketone*	500	38	0
Ethyl-*p*-guanidino-benzoate	140	30	11

Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 3×10^5 cpm) were prepared as described in Methods. The complete system consisted of HF from RSV-transformed CEF cultures supplemented with chicken serum at a final concentration of 2.5% (vol/vol) in a volume of 1 ml. To test the effect of inhibitors, HF was first mixed with the inhibitor and the serum added to start the reaction. The plates were incubated for 1 h at 37°C.

* Commercial samples that were used directly with no test or characterization.

of macromolecular soybean trypsin inhibitors powerfully inhibit the fibrinolysin, and the small molecules NPGB and ϵ-aminocaproic acid do likewise. Thus some step of the overall fibrinolytic activity appears to possess trypsin-like specificity. Fibrinolytic activity from RSV-transformed culture HF supplemented with chicken serum is unaffected by 10 mM EDTA, but is completely inhibited by 10 mM dithiothreitol.

Miscellaneous Observations on Fibrinolysin.—

Formation of cell factor does not represent simple leakage of cell constituents: It has been reported that transformed cells are "leakier" than normal fibroblasts and lose more of their intracellular constituents to the medium (24). It this were in fact the case, then the cell factor found in HF might be nothing more than a normal intracellular hydrolase that is released by transformed but not by normal cells. To test for this the proteins of normal and transformed cells were labeled by incubating cultures with [^{3}H]leucine for 24 h; the cultures were then washed and incubated under exactly the conditions used for producing HF. As shown in Table X, both normal and transformed cultures "leak" very little of the macromolecular constituents (less than 3% during the 12 h incubation) and the leakage by transformed cells is no greater than that of normal cells. Thus, under the conditions of our experiments, the transformed cells do not release more intracellular proteins to the medium than do normal cells, and the production of cell factor is not the result of nonspecific permeability differences.

Cell factor association with purified RSV virions: To test for the possibility that a component of RSV virions might be a participant in the fibrinolytic reaction, purified virus (6) was incubated both alone and with growth media under the conditions used for assaying HF. As seen in Table XI, both intact virions and virions disrupted by the nonionic detergent Triton X-100 show slight activity when assayed for cell factor. However, the viral content of factor is very low; in comparison with that of crude HF, the specific activity of virions is lower by a factor of >200. This low level of activity suggests that virions are intrinsically free of cell factor, and that the observed activity arises from the presence of small residual traces of contaminating cell factor that have accompanied the virus during purification.

A further indication that virions do not account for a significant proportion of the cell factor activity present in HF is given by the fact that there is no change in the titer of cell factor in supernatant HF after virions are removed by centrifugation at 100,000 *g* for 1 h.

Formation of cell factor is not associated with significant charges in lysosomal enzymes: To exclude the possibility that increased levels of lysosomal enzymes could account for the fibrinolytic activity of transformed cells, a number of lysosomal hydrolases were assayed in extracts of normal and RSV-infected CEF. Crude homogenates, freed of nuclei and unbroken cells, were assayed for a series of enzymes and the results (Table XII) show that with the exception of

cathepsin A, there are no significant differences between normal and transformed cells. The increased level of cathepsin A in transformed cultures is too small to account for the differences in fibrinolytic activity; moreover, cathepsin A is an unlikely candidate for the fibrinolysin since the pH optima of the two

TABLE X

Release of Protein by Normal and RSV-Transformed Chick Embryo Fibroblasts

Cells	Medium	Protein per plate	Total radioactivity incorporated	TCA-insoluble radioactivity in medium	Cellular release of protein (percent of total)
		μg	*cpm*	*cpm*	%
RSV-CEF	Medium + fetal bovine serum (10%)	421	4.6×10^5	7.3×10^3	1.6
	Medium + chicken serum (2.5%)	490	6.0×10^5	1.3×10^4	2.1
	Medium	386	5.6×10^5	1.2×10^4	2.1
Normal CEF	Medium + fetal bovine serum (10%)	405	5.6×10^5	7.6×10^3	1.4
	Medium + chicken serum (2.5%)	450	6.2×10^5	1.3×10^4	2.1
	Medium	330	5.7×10^5	1.2×10^4	2.1

RSV-transformed and normal CEF were plated at a density of 5×10^5 cells per 60 mm Petri dish. After 24 h the medium was removed and replaced with fresh medium containing fetal bovine serum (10%) and [^{3}H]leucine (20 μCi/ml). After 24 h the [^{3}H]leucine medium was removed, the plate washed with PBS, and the cells were incubated with medium as noted. After 12 h the media were centrifuged at 10^5 *g* for 1 h to remove the RSV virions produced by the infected and transformed cultures. The 5% TCA-precipitable radioactivity was measured in the supernatant after addition of bovine serum albumin (50 μg/ml) as carrier. Each value represents the average of two cultures. The cellular proteins were dissolved in 1 N NaOH after washing the monolayer with TCA (5%) to remove soluble constituents and radioactivity. Protein was measured by the method of Lowry et al. (18).

activities are widely separated (pH 5.2 for cathespin A, compared with pH 8.1 for purified fibrinolysin).

Formation of cell factor by primary cultures of viral chicken sarcomas: The physiological significance of the fibrinolysin for tumor biology would be questionable if the activity were not formed by tumor cells removed from intact fowl. We therefore infected 11-wk old cockerels in the wing web with the same

Schmidt-Ruppin virus (2×10^5 FFU) used for experiments in culture. Tumors were removed after 8 wk and the cells dispersed and placed into culture under standard conditions. The cell-free supernatant HF obtained from such primary cultures had high fibrinolytic activity when mixed with an activating serum such as chicken serum, and the same spectrum of inhibitory sera was seen as

TABLE XI

Fibrinolytic Activity of Native and Detergent-Disrupted RSV Virions

Sample tested	Protein	Additions	Radioactivity released into supernatant (percent of total)	
			1 hr	5½ h
	μg		%	%
Virus	300	None	1.2	5.3
	300	Chicken serum (2.5%)	1.3	42
	300	Triton X-100 (0.01%)	1.4	6.1
	300	Triton X-100 (0.01%) + Chicken serum (2.5%)	1.8	40
Controls		None	1.1	1.9
		Chicken serum (2.5%)	1.3	2.2
		Triton X-100 (0.01%)	1.4	1.6
		Triton X-100 (0.01%) + Chicken serum (2.5%)	1.2	2.2
RSV − HF	25	None	2.4	
	25	Chicken serum (2.5%)	25	
	25	Triton X-100 (0.01%) + Chicken serum (2.5%)	28	

Petri dishes (35 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 2.3×10^4 cpm per dish) were prepared as described in Methods. Purified virus was prepared as described previously (6). Virus was disrupted using 0.1% Triton X-100; aliquots (0.1 ml) of virus before and after detergent treatment were added to serum-free medium (final volume 1 ml) supplemented with chicken serum as noted, and incubated at 37°C. Controls not included show that sucrose added with the virus had no effect on enzyme activity. Proteins were determined by the method of Lowry et al. (18).

with RSV-infected CEF. Supernatant fluid obtained from minced Rous sarcoma tissue, stirred in Eagle's medium with 5% chicken serum for 3 h, released 41% of the radioactive [^{125}I]fibrin from plates under conditions in which only 0.02% was released by similar amounts of fluid obtained in the same way from normal wing tissue.

Normal chick fibroblasts do not secrete inhibitor of fibrinolysin or of transformed cell factor: The failure of HF from normal cultures to generate fibrinolysis with appropriate sera could, in principle, result from the secretion of an excess

of some inhibitor. Such an inhibitor might block either the interaction of cell and serum factors, or the subsequent fibrinolytic activity. We have attempted to test for the existence of such an inhibitor by mixing different proportions of normal and transformed HF before assay for fibrinolysin. Even with a twofold excess of normal HF (in terms of protein), no significant inhibition (less than 15%) of the rate of fibrinolysis was observed. Thus, little if any specific inhibitor is produced by normal cultures under these conditions.

An inhibitor in the sera of infected birds: If the fibrinolytic enzyme activity

TABLE XII

Lysosomal Enzyme Activities in Normal and RSV-Infected CEF

	Time after plating	Cells per 100 mm plate	Extracted protein per cell	*N*-acetyl glucosaminidase	β-Galactosidase	Cathepsin A	Acid phosphatase
	days		*pg*				
RSV-infected CEF	1	3.2×10^6	104	0.32	0.20	0.65	0.58
Normal CEF	1	1.8×10^6	189	0.30	0.19	0.61	0.53
RSV-infected CEF	2	6.3×10^6	128	0.34	0.17	0.58	0.50
Normal CEF	2	3.2×10^6	177	0.29	0.14	0.43	0.45
RSV-infected CEF	4	22×10^6	162	0.42	0.20	0.98	0.58
Normal CEF	4	12.5×10^6	182	0.36	0.19	0.57	0.49

Petri dishes (100 mm diameter) were inoculated with either 2×10^6 normal or 2.2×10^6 RSV-infected CEF, respectively. After incubation for the indicated length of time, the monolayers were typsinized and the cells harvested, counted and then homogenized as described in Methods. Activities are expressed as micromoles of substrate hydrolyzed per milligram protein per hour.

observed in virus-transformed cultures and in primary cultures of chicken sarcomas were an important element in the tumor life cycle in intact animals, it is possible that some natural defense mechanism might be present to combat its activity. This possibility prompted us to compare the sera of normal and tumor-bearing cockerels for the ability to produce measurable fibrinolysin when mixed with HF from transformed cultures. As seen in Table XIII, the limited results indicate that the sera of tumor-bearing fowl do not activate the enzyme; this is in contrast to the sera of normal fowl. We have so far examined the individual sera of 11 normal and 12 tumor-bearing birds: all of the normal sera are activating when mixed with transformed cell HF, and all of the sera from tumor-bearing birds are nonactivating. Also given in Table XIII are the results of experiments in which the sera of normal and tumor-bearing animals are

mixed; these show progressive inhibition of fibrinolytic activity as the proportion of serum from tumor-bearing birds is increased. This indicates that the serum from tumor-bearing birds contains an inhibitor of the fibrinolysin and encourages the expectation that the enzyme activity may be expressed under conditions of tumor growth in vivo.

TABLE XIII

Fibrinolytic Activity of HF from RSV-Infected Culture: Comparison of the Effects of Sera from Normal and RSV Tumor-Bearing Chickens

Test chicken	Test chicken serum final concentration	Normal chicken serum*	Radioactivity released (percent of total)
	%	%	%
	0	5.0	65
	0	2.5	61
Normal chicken‡	2.5	2.5	43–63§
III–XIV	2.5	0	36–60§
RSV-tumor bearing chickens‡			
XII	10	0	1.2
	0	10	85
	7.5	2.5	4.2
	10	2.5	1.3
	0	2.5	70
IX	10	2.5	1.5
	10	0	0.37
X	10	2.5	1.6
	10	0	1.9
XI	10	2.5	0.2
	10	0	0.2

Petri dishes (35 mm diameter) containing [^{125}I]fibrinogen (10 μg/cm^2; total radioactivity 3×10^4 cpm) were prepared as described in Methods. The appropriate sera were added to HF from RSV-transformed CEF cultures to yield the indicated final concentrations in a final volume of 1 ml. The plates were then incubated for 5 h at 37°C.

* Obtained from GIBCO.

‡ SPAFAS.

§ These values represent the range of activities given by sera from 12 individual normal cockerels.

The failure of sera from tumor-bearing fowl to activate fibrinolysis under cell-free conditions with HF is parallelled by their effects in cultures of transformed cells; these results are given in Table XIV.

SUMMARY

Chick embryo fibroblast cultures develop fibrinolytic activity after transformation by Rous sarcoma virus (RSV). This fibrinolytic activity is not present in normal cultures, and it does not appear after infection with either nontrans-

forming strains of avian leukosis viruses or cytocidal RNA and DNA viruses. In cultures infected with a temperature sensitive mutant of RSV the onset of fibrinolysis appears after exposure to permissive temperatures and precedes by a short interval the appearance of morphological evidence of transformation.

TABLE XIV

Fibrinolytic Activity of RSV-Transformed CEF: Effects of Sera from Normal and RSV Tumor-Bearing Chicks

Serum	Final serum concentration	Radioactivity released into growth medium (percent of total)
	%	
Chicken*	2	21
	5	32
Normal chickens‡		
I	2	11
II	2	11
III	2	12
RSV tumor-bearing chickens‡		
I	2	4.4
	5	1.6
II	2	1.5
	5	1.8
III	2	3.2
	5	3.7
IV	5	4.1
V	5	3.8
VI	5	3.2
VII	5	4.5
VIII	5	2.5

Petri dishes (60 mm diameter) containing [^{125}I]fibrin (10 μg/cm^2; total radioactivity 8×10^4 cpm) were prepared as described in Methods, and inoculated with RSV-transformed CEF at a density of 5×10^5 cells per dish. After 24 h growth in media containing fetal bovine serum at a concentration of 10%, the monolayers were washed carefully with TD, and fresh medium containing the indicated serum supplements was added. The cultures were then incubated for 5 h, and aliquots of the growth medium assayed for radioactivity.

* GIBCO.

‡ SPAFAS.

The rate of fibrinolysis in transformed cultures depends on the nature of the serum that is present in the growth medium: some sera (e.g., monkey or chicken serum) promote high enzymatic activity, while others (calf, fetal bovine) do not. Some sera contain inhibitors of the fibrinolysin.

Based on the effect of a small number of known inhibitors, at least one step of the fibrinolytic process shows specificity resembling that of trypsin.

The sera of sarcoma-bearing chickens contain an inhibitor of the fibrinolysin, whereas normal chicken sera do not.

For general discussion, conclusions, and summary see the accompanying paper, part II, (*J. Exp. Med.* **137**:112).

REFERENCES

For references, see the following paper, part II.