Deoxyribonuclease-Labile Infective Units from Adenovirus

In 1961, SMULL, MALLETTE, and LUDWIG[1] introduced the isotonic histone method for infecting HeLa cells with poliovirus RNA. A variation on this theme appeared in 1965, when PAGANO and VAHERI[2] reported that another polycation, diethylaminoethyl (DEAE)-dextran, enhanced infection with poliovirus RNA. The purpose of this report is to describe our finding that DEAE-dextran enhances infection of human carcinoma strain KB cells with heat-shocked (70 °C, 20 sec) human adenovirus type 1.

Materials and methods. Cells, media and plaquing. Calf serum-adapted KB cells, from the American Type Culture Collection (ATCC), were grown and maintained under medium S, which contains 4% calf serum, except for short-term maintenance under medium H, which is medium S without serum (CHAPIN and DUBES[3]). Cells were washed with (1) the phosphate-buffered saline (PBS) of DULBECCO and VOGT[4], (2) PBS minus $CaCl_2$ and $MgCl_2$, referred to as medium A, or (3) the balanced salt solution (BSS) of HANKS and WALLACE[5]. A modification of the plaque method of ROUSE, BONIFAS, and SCHLESINGER[6] was used.

Viruses. The Adenoid 71 strain of human adenovirus type 1, from ATCC, was used. Most of the experiments were done with a large-plaque mutant (DUBES, ROEGNER and MOYER, unpublished) of this strain. Virus stocks were prepared in PBS-washed infected cells under medium H and gave titers usually $1–4 \times 10^8$ plaque-forming units (pfu)/ml.

Heat-shock. In most tests, a sample of virus stock was diluted 6- or 20-fold into 10 m*M* Na_2HPO_4 at the 'shock' temperature. After a predetermined time, a sample was diluted into 10 m*M* Na_2HPO_4 at 0 °C. In a few tests, the virus stock was heated undiluted. In all tests, care was taken that no microdroplet escaped the heating.

DEAE-dextran enhancement method. The cell sheet in a 60 mm Petri dish was covered with 5 ml medium A and incubated at about 25 °C for 15 min. The medium A was discarded, and the plate was inoculated with heat-shocked virus in medium A containing DEAE-dextran (Pharmacia, M.W. 2×10^6) at 300 μg/ml and Na_2HPO_4 at 7 or 8 m*M* (from the heating and cooling diluent). Inoculum volume was usually 0.2 or 0.3 ml. The inoculated dishes were incubated at about 25 °C for 15 min, washed with 5 ml BSS, and overlayered for plaque development.

Enzymes, inhibitor and sera. Electrophoretically purified deoxyribonuclease I was obtained from Sigma Chemical Co. Bovine pancreatic ribonuclease (crystallized 5 times, salt free) and trypsin (crystallized twice) were obtained from Mann Research Laboratories, Inc. Solutions containing 500 μg enzyme/ml prepared in autoclaved deionized distilled water were passed through Nalgene membranes of 450 nm porosity and stored at − 60 °C. A solution of soybean trypsin inhibitor (crystallized 5 times, Nutritional Biochemicals Corp.) at 2.5 mg/ml prepared in autoclaved deionized distilled water was passed through an ultrafine sintered glass filter and stored at − 20 °C. Human adenovirus type 1 antiserum and control serum, both from rabbits, were obtained from Microbiological Associates.

Results. Infectivity after heat-shock. When DEAE-dextran was used, plaques were regularly produced after inoculating virus shocked at 60°, 70° or 80 °C for 20 sec. When phenol was used in place of heat-shock, no infectivity was detected. Higher titers were obtained when the virus stock was heat-shocked in a 6- or 20-fold dilution than when it was heat-shocked undiluted. When the DEAE-dextran enhancement method was followed, but the DEAE-dextran itself was omitted, no plaques were produced. The number of plaques produced by 70 °C-shocked virus was (a) largely independent of the time at 70 °C (Figure 1) and (b) directly proportional to the concentration of heated virus inoculated (Table I). The infectivity was stable in 10 m*M* Na_2HPO_4 at 0 °C for at least 2 h (Figure 2).

Effects of enzymes. Incubation of 70 °C-shocked virus with deoxyribonuclease or trypsin destroyed the infectivity. That each enzyme was effective because of its action on the infective units rather than on the infection process was shown by tests including additions of enzymes to the inocula just before inoculation (Table II). For

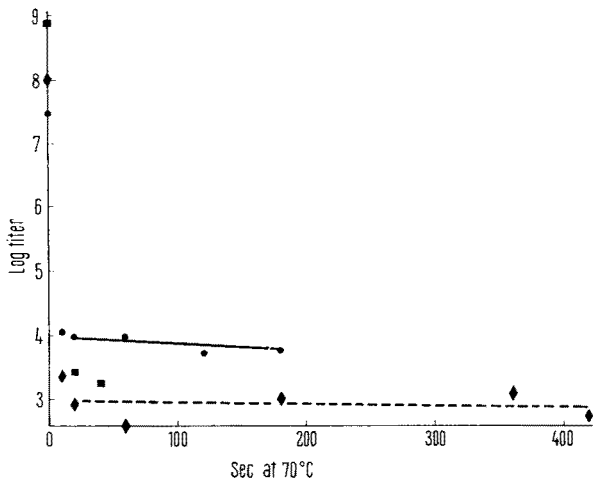

Fig. 1. Effect of time at 70 °C. Log titer is $\log_{10}$ (pfu/ml), calculated back to undiluted virus stock in each case. The data are from 3 experiments, each with a different virus stock and a different cell batch. Circles and diamonds, experiments heating virus stock 20-fold diluted in 10 m*M* Na_2HPO_4; squares, experiment heating undiluted stock.

Table I. Plaquing linearity

Concentration of 70 °C-shocked stock inoculated	Mean number of plaques/Petri dish		
	Experiment C32	Experiment C41	Experiment C66
1/100	not done	not done	38
1/30	20	38	not done
1/10	62	116	400

[1] C. E. SMULL, M. F. MALLETTE and E. H. LUDWIG, Biochem. biophys. Res. Commun. *5*, 247 (1961).
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[4] R. DULBECCO and M. VOGT, J. exp. Med. *99*, 167 (1954).
[5] J. H. HANKS and R. E. WALLACE, Proc. Soc. exp. Biol. Med. *71*, 196 (1949).
[6] H. C. ROUSE, V. H. BONIFAS and R. W. SCHLESINGER, Virology *20*, 357 (1963).

trypsin, this point was confirmed using soybean trypsin inhibitor; and the effectiveness of this inhibitor showed that the effect of the trypsin preparation was due to trypsin itself (Table III). Incubation of 70 °-shocked virus with ribonuclease did not significantly affect the infectivity.

Inactivation by $MgCl_2$. In early tests of deoxyribonuclease, $MgCl_2$ was added. Controls soon established that $MgCl_2$ by itself abolished the infectivity of 70 °C-shocked virus and that the $MgCl_2$ was effective because of its action on the infective units (Table IV).

Non-neutralizability. Incubation of 70 °C-shocked virus in 0.25% (by volume) antiserum did not affect its infectivity; this concentration completely neutralized samples of unheated virus (Table V).

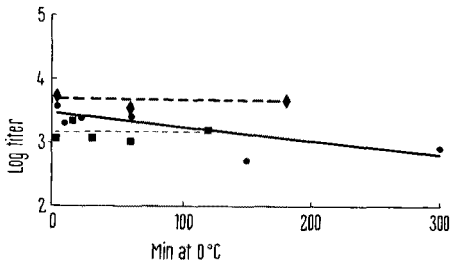

Fig. 2. Effect of time at 0 °C after 70 °C-shock. Log titer as in Figure 1. Data are from 3 experiments, with different virus stocks and cell batches. Log titers of unheated samples were: circle experiment, 7.9; diamond, 7.8; square, 8.2. During incubation, the virus stock was at a concentration of 1/80 (circles and diamonds) or 9/80 (squares).

Table II. Action of deoxyribonuclease and trypsin

Incubation at 15 °C[a]	Late enzyme addition	Mean number of plaques/Petri dish
No added enzyme	none	18
	deoxyribonuclease[b]	22
	trypsin[b]	16
Deoxyribonuclease, 3 μg/ml	none[b]	0.3
Trypsin, 3 μg/ml	none[b]	1.8

[a] All incubated, with virus stock at concentration 9/10, for 20 min. [b] Added enzyme at 1/3 μg/ml in inoculum.

Table III. Action of trypsin inhibitor

Concentration of added enzyme during 5-min incubation of 70 °C-shocked virus at 15 °C	Subsequent pre-cell incubation with soybean trypsin inhibitor (SBTI)	Mean number of plaques/Petri dish[b]
No added enzyme	no	23
	yes[a]	20
Trypsin, 20 μg/ml	no	0
	yes[a]	0
Trypsin, 20 μg/ml, pretreated with SBTI[a]	no	16

[a] Incubation with SBTI, at concentration (in μg/ml) 8 times that of trypsin, at 15 °C for 30 min. [b] 4 or 5 dishes for each inoculum.

Discussion. The plaquing linearity suggests that each infective unit present after 70 °C-shock is sufficient for plaque initiation. Denaturation of capsid proteins is extremely rapid at 70 °C; yet the effect of trypsin suggests that some protein is essential to the infective unit 'surviving' this temperature, and the plaquing linearity further suggests that this essential protein is part of the infective unit. Thus, we view this infective unit as exposed adenovirus DNA with some protein, presumably capsid, attached. The essential function of this protein is not clear, though it is clear that its function is not neutralizable with specific antiserum. One hypothesis is that the protein performs a compacting service; that is, it may diminish the extension of the DNA and thus make more likely the presentation of a manageable DNA package to the cell. In this regard, we recall that extended adenovirus DNA is 11–13 μ long (GREEN, PIÑA, KIMES, WENSINK, MACHATTIE, and THOMAS[7]), which is about half the distance across a KB cell.

That $MgCl_2$ inactivates the infective unit is reminiscent of the inactivating effect of Mg^{++} on poliovirus RNA (LAMB, CHAPIN, and DUBES[8]). However, though Mg^{++} is

Table IV. Effect of $MgCl_2$

Concentration (mM) of added $MgCl_2$ during incubation at 15 °C[a]	Late $MgCl_2$ addition	Mean number of plaques/Petri dish[d]
0	no	3.7
	yes[b]	5.8
	yes[c]	4.8
10	no[b]	0.5
25	no[c]	0

[a] All incubated, with virus stock at concentration 9/80, for 20 min. [b] Added $MgCl_2$ at 0.89 mM concentration in inoculum. [c] Added $MgCl_2$ at 2.2 mM concentration in inoculum. [d] 4 dishes/inoculum.

Table V. Effect of sera

Virus	Experiment	Mean number of plaques/Petri dish[a] after incubation[b]		
		Without serum	With 0.25% control serum	With 0.25% antiserum
Unheated control	1	49	44	0
	2	26	23	0
70 °C-shocked	1	70	83	60
	2	34	25	29

[a] 3 or 4 dishes/inoculum, for each experiment. [b] At 15 °C for 30 min.

[7] M. GREEN, M. PIÑA, R. KIMES, P. C. WENSINK, L. A. MACHATTIE and C. A. THOMAS JR., Proc. natn. Acad. Sci., USA *57*, 1302 (1967).
[8] R. D. LAMB, M. CHAPIN and G. R. DUBES, Arch. ges. Virusforsch. *15*, 486 (1965).

known to catalyze chain scission of RNA (e.g. LINDAHL[9]), the action of Mg^{++} on DNA is probably different (e.g. LYONS and KOTIN[10]). LYONS and KOTIN indicated that an excess of $MgCl_2$ could cause charge reversal of calf thymus DNA and that large charge reversal would destabilize the 2-stranded structure. Also, the mere changing of adenovirus DNA from a polyanion to a polycation by adding Mg^{++} might give a product whose infectivity would not be enhanced by a polycation like DEAE-dextran[11].

Zusammenfassung. Diäthylaminoäthyl Dextran erhöht die Infektion menschlicher Krebszellen (Stamm KB) mit hitzebehandelten (70 °C, 20 sec) menschlichen Adenoviren, Typ 1, stark. Inkubation dieser hitzebehandelten Viren mit Desoxyribonuklease, Trypsin oder $MgCl_2$ (nicht mit Ribonuklease oder spezifischem Antiserum) konnte die Infektiosität leicht beseitigen.

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[9] T. LINDAHL, J. biol. Chem. *242*, 1970 (1967).
[10] J. W. LYONS and L. KOTIN, J. Am. chem. Soc. *87*, 1781 (1965).
[11] Acknowledgment: We thank H. NELSON and KAREN HANGREN for skilled assistance.

Formation of Abnormal Mycelium of *Fusarium roseum* Link. on a Modified Czapek-D-Amino Acid Medium

In the previous reports[1,2], the authors have mentioned that some of plant pathogenic fungi have an ability to utilize D-amino acid. The present study was undertaken to investigate the morphological characteristics of the mycelium which developed meagerly on a medium containing D-amino acid as a nitrogen source.

G-strain (Shinshu University) of *Fusarium roseum* Link. was inoculated on a modified Czapek's medium in which $NaNO_3$ was replaced by an equivalent nitrogen content of D- or L-isomer of 9 amino acids, and then kept at 28 °C.

After 10 days, the mycelium was examined under a light microscope; the mycelium which developed poorly on a modified Czapek's medium containing D-alanine, D-arginine, D-lysine or D-methionine, is broader in width than that of the respective L-amino acid, and has many large granules in the cell.

Figures 2 and 3 show a yeast-like cell of the mycelium developed on the medium containing D-arginine, on which the growth was retarded. The morphological development in the plot of L-alanine, L-arginine, L-lysine or L-methionine was the same as that in Czapek ($NaNO_3$) medium. It was evident, however, that yeast-like cells of mycelia were observed on the medium containing D-arginine, and chlamydospore-like cells were found on the medium containing valine, especially its D-isomer, as shown in Figure 4. The yeast-like cells were found after 4 days on the Czapek-D-arginine medium. These cells are clearly different from the chlamydospore.

To clarify the effect of D-arginine concentration on the formation of this yeast-like cell, D-arginine was diluted to $^1/_2$, $^1/_4$, $^1/_8$ and $^1/_{16}$ from a modified Czapek's basal medium. After 10 days, a lot of yeast-like cells was observed in all the plots of D-arginine concentration up to $^1/_8$. But, in the plot of $^1/_{16}$ D-arginine, the formation was poor.

To know the effect of D/L ratio of amino acid in Czapek's medium on the formation of yeast-like cells, the ratio of D-arginine to L-arginine was varied to (6:0), (5:1), (4:2), (3:3), (2:4), (1:5) and (0:6) respectively. After 10 days, abnormal mycelia were observed abundantly in the plots of higher ratio, (6:0) and (5:1). The formation was poor in (4:2) or (3:3) and no formation in (2:4), (1:5) or (0:6).

In this connection, it would be interesting to study the metabolism of D-amino acids, especially D-arginine by plant pathogenic fungi in view of the morphogenesis in fungi[3,4]. Details will be reported elsewhere.

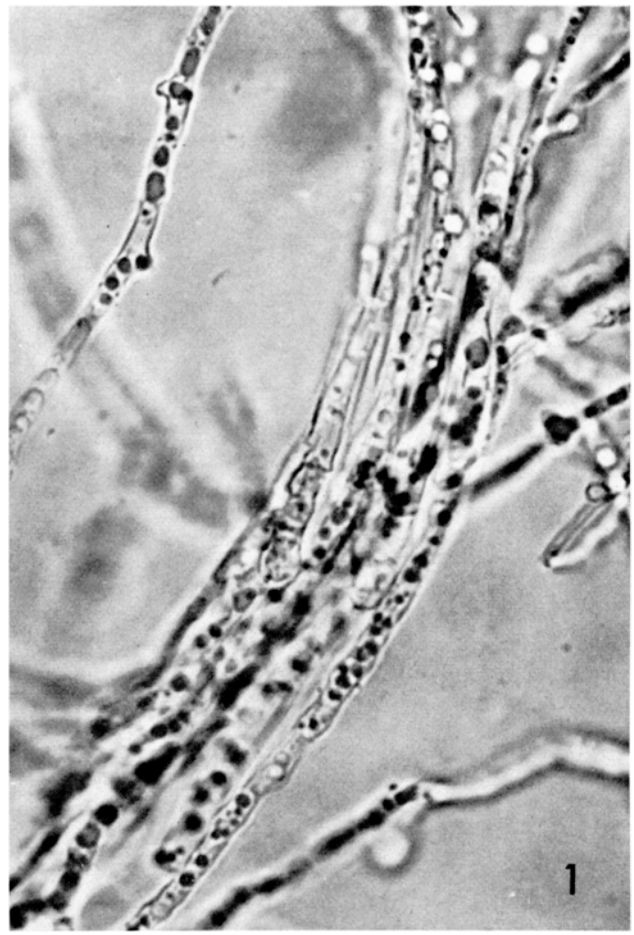

Fig. 1. Mycelia of *F. roseum* obtained from a Czapek-L-arginine medium. Ca. × 400.