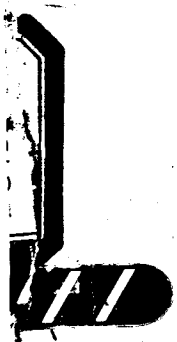
THE BIOLOGY OF ONCOGENIC VIRUSES

L. G. SILVESTRI



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Proceedings of the second Lepetit Colloquium,
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WELCOMING ADDRESS

Ladies and Gentlemen,

It is a particular pleasure for me to welcome all of you who have accepted our invitation to participate in the Second Lepetit Colloquium.

As some of you already know, our First Colloquium had the character of an experiment. We should have continued only if the results were satisfactory. In fact our First Colloquium was much more of a success than we expected. So we were willing to continue and transform what was an experiment into a tradition.

But if a good start is encouraging, to maintain the same high level becomes something of a problem.

The choice of subject for the first meeting was not difficult and did not require any particular insight as to the progress of biological research. As discoverers of the rifamycins, transcription was a natural choice.

The time was ripe. Our only merit was to have obtained the collaboration of the best people. But this year's choice was less simple.

We wanted a subject in which no general consensus of opinion had yet been established, but whose progress towards an understanding could be speeded up by such a meeting.

Maybe it is a little early to say this, but I have the impression that we have achieved our goal on this occasion as well.

Since the end of the Spring tumor viruses have stolen the show and such an authoritative journal as Nature has been able to publish a headline "Apres Temin, le déluge". But when we took the decision to select tumor viruses, we were only making a guess and were not aware that rifamycins were to play an important role also in this field.

We must thank our advisers for their suggestions, and particularly Prof. S. Spiegelman, G. Di Mayorca and many others whose suggestions have helped in overcoming our hesitations. I also want to thank our convenors, R. Dulbecco, A. Lwoff, H. Hanafusa, J.E. Darnell, M.G.P. Stoker, G. Di Mayorca and E. Winocour, who suggested the names of the invited speakers.

Of course, some omissions might be remarked on, some valuable contributors are probably missing. But it is human not to be perfect. On the other hand, we have to recognize that our convenors have invited here a group which is fully representative of the subject in its present state.

Some papers have been admitted which are not strictly dealing with the present subject such as a paper on the effect of a new derivative of rifampicin on ribosomal DNA amplification in amphebian oocytes.

Maybe in the next years we shall consider viral integration as a sort of specialized infectious gene amplification.

But before I invite the first Chairman, let me thank the Institut Pasteur, and particularly Dr. Elie Wollman who has so kindly given hospitality to our Colloquium.

Finally let me thank all the speakers who in the next three days will let us enjoy hearing their interesting results.

One more word about the absentees. Dr. J. Svoboda and Dr. J. Riman from Prague and Dr. G.I. Deichman from Moscow, who, although originally willing to participate, were not able to come for reasons "which are beyond their control", as one of them was good enough to explain me. We can only complain about this bureaucratic and/or political obtuseness. We can only look to that day when eventually there will be one science in one world.

May I now ask Prof. Lwoff to take the chair.

Luigi G. Silvestri
Gruppo Lepetit S.p.A.
Director of the Department
of Microbiology

OPENING

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As some of you might perhaps know, this symposium is devoted to the biology of oncogenic viruses.

Since the RNA–DNA affair, some of them have become more and more molecular, that is more and more fashionable. As a consequence, competition has reached an unprecedented level. This is unfortunately not all.

Infectious particles possessing only one type of nucleic acid belong to the category Virus. There is no exception to this rule. However, it is not true any more that the possession of only one type of nucleic acid is one of the discriminative characters of viruses. It is fortunate that viruses have not been defined by this character only. A number of others are still available. The infectious particle is unable to grow, unable to divide and is reproduced from its genetic material alone. It does not possess ribosomes. It is devoid of the information for the synthesis of transfer RNA, of ribosomal RNA and also for the production of energy metabolism enzymes. The category Virus can thus be defined without ambiguity. Until 1970, all the viral features were correlated – including the possession of only one nucleic acid. Today, the virions possessing two types of nucleic acid are the exception which confirm the rule.

The RNA–DNA turmoil had another consequence. Almost all classifications of viruses start by separating DNA viruses from RNA viruses. Today, a third group has to be created to take care of viruses with the two types of nucleic acids.

The astuteness of living-beings is infinite. A good classification has to be flexible and able to cope with their perversity.

Of course, as in every decent meeting of this time, you will hear of repression, induction, transformation, phenotype, reversion and mutation.

To close this brief introduction, I would like to express the hope that it would be well not to forget that oncogenic viruses sometimes, not always, produce cancer.

CONTENTS

STUDY ON THE MODE OF INHIBITION BY SV 40 'REPRESSOR' OF PRODUCTIVE SV 40 VIRUS INFECTION

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Abstract: Extracts from simian virus 40 (SV 40) transformed hamster, mouse, or cat cells contain a protein(s), termed "repressor", which specifically inhibits SV 40 plaque formation in monkey cells by approximately 50 %.

The mode of action of the "repressor" extracts is being studied:

(i) the total yield of virions in monkey (CV-1) cells infected with either SV 40 virus or viral DNA is reduced by 50 % in the presence of "repressor".

(ii) viral DNA extracted by the Hirt procedure from CV-1 cells treated with "repressor" after infection with SV 40 virus is 50 % less infectious than viral DNA extracted from control cultures. Gross synthesis (as measured by incorporation of ^{3}H-thymidine), size and shape of the viral DNA are not affected. Infectivity could be recovered by treatment of the viral DNA with trypsin.

(iii) synthesis of SV 40 tumor (T) antigen is not appreciably affected, while that of the structural (V) antigen is reduced by approximately 40 %.

1. INTRODUCTION

In animal cells transformed by simian virus 40 (SV 40) - even those in which a complete viral genome is present - some viral gene products (tumor T and transplantation antigens) are produced, but autonomous replication of viral DNA and synthesis of viral structural (V) antigens are usually not detected (Black, 1968; Kit, 1969; Green, 1970). Therefore, the expression of selected viral genes is blocked. This phenomenon may be due to specific repression.

We have previously shown (Cassingena and Tournier, 1968; Cassingena et al., 1969a) that extracts of SV 40 transformed hamster, mouse or cat cells contain a protein(s) - termed "repressor" - which specifically inhibits SV 40 plaque formation in monkey cells by approximately 50 %.

The present report describes a study on the mode of action of SV 40 "repressor" extracts.

2. MATERIALS AND METHODS

(i) Cells : two different established cell lines were used in

this study : CV-1, green monkey kidney cells (Jensen et al., 1964), and EHSVi, hamster embryo cells transformed by SV 40 virus (Tournier et al., 1967).
The cells were cultivated in modified Eagle's medium supplemented with 10 % calf serum (Tournier et al., 1967).

(ii) Virus : SV 40 virus (strain Hilleman) was grown and assayed by plaque formation in monolayer cultures of CV-1 cells (Tournier et al., 1967).

(iii) Viral DNA purification : Using the selective procedure devised by Hirt (1967), viral DNA was extracted from SV 40 virus infected CV-1 monolayer cultures labeled with ^{3}H-thymidine (New England Nuclear Corp., Boston, Mass.; specific activity 15 C/mmole). Form I viral DNA was obtained by nitrocellulose column chromatography (Kit et al., 1967). Phenol (1/20 volume of 90 % redistilled) was added to the DNA and the solution was dialysed against cold 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7). The infectivity of the DNA was determined by plaque titration on monolayer cultures of CV-1 cells (Kit et al., 1968).

(iv) Preparation of poly-L-ornithine solution : As described previously (Cassingena and Tournier, 1968; Cassingena et al., 1969a), a basic polymer was needed with "repressor" extracts to observe viral inhibition presumably because it induces pinocytosis (Ryser, 1967) and thereby enables the "repressor" to enter the cells. Poly-L-ornithine (M.W. 90,000; Sigma, St-Louis, Mo.) was used. Stock solutions, prepared by dissolving 1 mg polymer per ml medium without serum, were stored at - 20°C.

(v) Preparation of "repressor" extracts : EHSVi cells were suspended in cold Tris buffer (0.01 M, pH 8) at a concentration of 5 x 10^{7} cells per ml. The cell suspension was subjected to ultrasonic treatment for 2-3 minutes at low frequency at 4°C (Siduse sonic oscillator, model US 77-5, Paris). Complete cell disintegration was monitored by microscopic examination. The clear supernatant ("repressor" extract; an average of 8 mg protein per ml), derived from centrifugation for 1 hr. at 37,000 rpm at 4°C (Beckman SW-65 rotor), was mixed with cold poly-L-ornithine solution (10 µg of polymer per ml supernatant). It was then diluted to the desired cell equivalent concentration in cold medium (without serum) containing 10 µg poly-L-ornithine per ml. Extracts were used immediately after preparation. It has been previously observed (Cassingena et al., 1969a) that, for a given number of monkey cells to be treated, the "repressor" extracts were more effective within a certain range of concentration : 0.62 to 2.50 cell equivalents of extract per treated cell (cell equiv. per treated cell). In the present experiments, this fact was always considered.

(vi) Effect of "repressor" extracts on the total yield of SV 40 virions in CV-1 cells :

a) Infection with virus : Confluent cultures of CV-1 cells (10^{5} cells per tube) were infected with 0.2 ml of SV 40 virus at a multiplicity of infection (m.o.i.) of 0.01, 0.1, 0.5 or 1 PFU per cell. After 2 hrs. adsorption at 37°C, unadsorbed virus was removed. Half of the infected cultures received 0.2 ml (1.25 x 10^{5} cell equivalent) of "repressor" extract, the other half was used for the control (medium without serum, containing 10 µg poly-L-ornithine per ml). After 30 minutes incubation at 37°C, the inoculum was discarded, the cells were washed, 1 ml of medium supplemented with 0.5 % calf serum was added and the cultures were reincubated at 37°C. At various time intervals, "repressor" treated and control cultures were frozen and thawed twice (- 70°C to 37°C), sonicated for 5 minutes at low frequency at 4°C, and assayed for plaque formation in monolayer cultures of CV-1 cells (6 Petri dishes per viral dilution).

b) Infection with viral DNA : Confluent cultures of CV-1 cells (10^5 cells per tube) were washed with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}. Purified SV 40 DNA was diluted to 5 x 10^4 PFU per ml in PBS (without Ca^{2+} and Mg^{2+}) containing 1 mg per ml DEAE-Dextran (Pharmacia, Uppsala); 0.2 ml of this solution was layered on top of each cell monolayer. After incubation for 30 minutes at room temperature, the inoculum was removed and the cells were washed with PBS. We then proceeded with "repressor" extract treatment as described in paragraph (a) above.

(vii) Characterization of viral DNA synthesized in the presence of "repressor" extracts :

a) Incorporation of ^{3}H-thymidine into viral and cellular DNA's : Confluent cultures of CV-1 cells (5 x 10^6 cells per flask) were infected with 1 ml of SV 40 virus at an input multiplicity of 0.05, 0.5 or 5 PFU per cell. After an adsorption period of 2 hrs. at 37°C, unadsorbed virus was removed. The cultures then received 1 ml of "repressor" extract (3.12 x 10^6, 6.25 x 10^6 or 12.50 x 10^6 cell equivalents); control cultures were treated simultaneously. After 30 minutes of incubation at 37°C, the inoculum was discarded, the cells were washed, 10 ml of medium supplemented with 2 % calf serum was added and the cultures were reincubated at 37°C. Twelve hours post infection, the cultures were inoculated with 0.1 ml solution containing 50 µC of ^{3}H-thymidine (Centre Energie Atomique, Gif sur Yvette; specific activity 18 C/mmole) and 25 µg of cold thymidine. At various times after infection, SV 40 DNA was extracted by Hirt's procedure. Most of the cellular DNA, complexed with SDS, is precipitated with NaCl; centrifugation results in a supernatant containing the viral DNA (Hirt extracts). The viral DNA extracts were mixed for about 1 minute with an equal volume of 90 % phenol (redistilled) and centrifuged at 10,000 rpm for 30 minutes at 24°C (I.E.C. International 856 angle rotor). They were then dialysed against cold 1 x SSC and stored at 4°C.

To determine radioactivity, 0.2 ml aliquots were solubilized with 1.5 ml of NCS reagent (Nuclear Chicago, Ill.), mixed with 10 ml of scintillation fluid (toluene-Liquifluor, Nuclear Chicago) and counted in an Intertechnique liquid scintillation counter ("ABAC", type SL 40, PLAISIR, France). To determine the amount of DNA in a given extract, a sample was treated with 0.2 N NaOH for 1 hr. at 37°C to degrade RNA and dialysed against 1 x SSC; the optical density of the dialysate was measured at 260 and 280 mµ. The O.D. ratio 260:280 ranged from 1.35 to 1.85.

The incorporation of ^{3}H-thymidine into cellular DNA was determined after perchloric acid hydrolysis of 5 % trichloroacetic acid (TCA) - washed pellets from the Hirt extraction.

b) Infectivity of the viral DNA extracts : The infectivity of the viral DNA extracts was determined, as described by Kit et al. (1968), by plaque titration on monolayer cultures of CV-1 cells (4 to 8 Petri dishes per extract dilution).

c) Neutral CsCl sedimentation gradients on extracted viral DNA : Band centrifugation, carried out as described by Weil et al. (1965), was performed on 0.2 ml of the DNA extracts (containing a maximum of 4 µg DNA) layered between 3 ml CsCl solution (density = 1.509 at 20°C) and 1.8 ml paraffin oil. The tubes were centrifuged for 2.5 hrs. at 37,500 rpm at 20°C (Beckman SW-65 rotor); eight drop fractions were collected from the bottoms of the tubes onto filter discs (N° 593 Schleicher & Schull, Dassel, Germany), dried and counted in 5 ml toluene-Liquifluor scintillation fluid as described above.

d) Alkaline sucrose gradients on extracted viral DNA : One-tenth ml of 0.22 N NaOH was layered on top of 4.4 ml gradients of 5-20 % sucrose (0.1 N NaOH, 0.9 N NaCl). One-tenth ml of the

DNA extracts was gently pipeted onto the gradients; after standing 15-20 minutes, they were centrifuged for 1.5 hr. at 35,000 rpm at 5°C. Fractions were collected and counted as described above.

e) Effect of trypsin on the infectivity of the viral DNA extracts : Viral DNA extracts obtained from an experiment performed with an m.o.i. = 0.5 PFU per cell were used. One-half ml of each sample was mixed either with 0.1 ml PBS containing 1000 µg per ml trypsin (2 x recrystalized; Nutritional Biochemicals Corp., Cleveland, Ohio) or with 0.1 ml PBS. After 30 minutes incubation at 37°C, the samples were quickly chilled and mixed with 0.1 ml Iniprol (20,000 UIP; Choay, Paris). Infectivity was determined by plaque titration (6 Petri dishes per sample dilution) as described above.

(viii) Effect of "repressor" extracts on the synthesis of SV 40 tumor (T) and structural (V) antigens in CV-1 cells :

Cultures of CV-1 cells growing on 12 x 32 mm cover slips in Leighton tubes (5 x 10^4 cells per cover slip) were infected with SV 40 virus (m.o.i. = 1 PFU per cell) and treated with "repressor" extracts (0.31 x 10^5, 0.62 X 10^5 or 1.25 x 10^5 cell equivalents) as described in paragraph 6a. Sixty three hours after infection

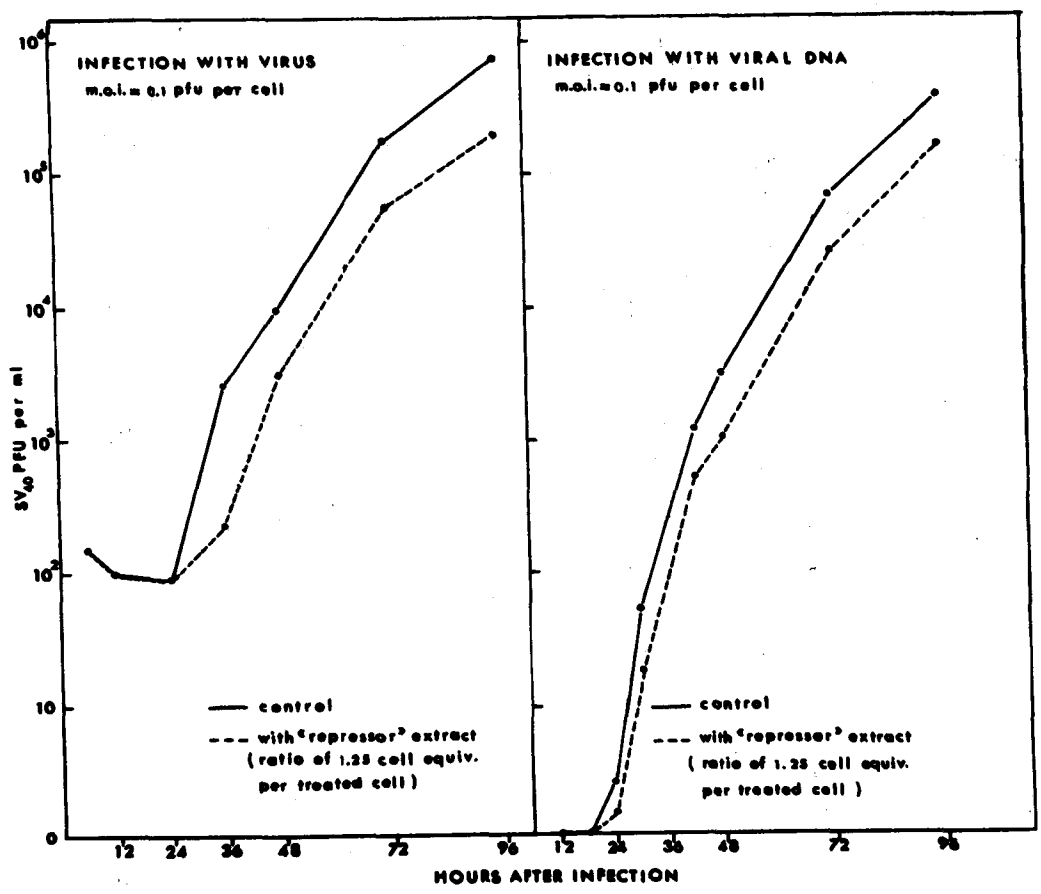

Fig. 1 - Total yield of virions in CV-1 cells treated with "repressor" extracts after infection with SV 40 virus or viral DNA.

the cover slips were withdrawn, washed three times with PBS, fixed with acetone for 8 minutes, dried and stored at -70°C. For the detection of the SV 40 tumor (T) and structural (V) antigens, both the fluorescein isothiocyanate (immunofluorescence) and the horseradish peroxidase staining procedures were employed (Wicker and Avrameas, 1969). The materials needed for these assays were kindly supplied by R. Wicker. For each procedure, 1000 cells were counted on each of 3 cover slips used per sample, and the average was calculated.

3. RESULTS

I. Effect of "repressor" extracts on total yield of SV 40 virions after infection with SV 40 virus or viral DNA.

The effect of "repressor" extracts on the kinetics of virion production in CV-1 cells infected with SV 40 virus at different multiplicities of infection (m.o.i. = 0.01, 0.1, 0.5, 1.0 PFU/cell) has been investigated. The results obtained with an input multiplicity of 0.1, shown in fig. 1, were similar to those observed with the other multiplicities.

The time course of production of virions in CV-1 cells treated with "repressor" extract after infection with SV 40 DNA (m.o.i. = 0.1 PFU of DNA/cell), is also shown in fig. 1.

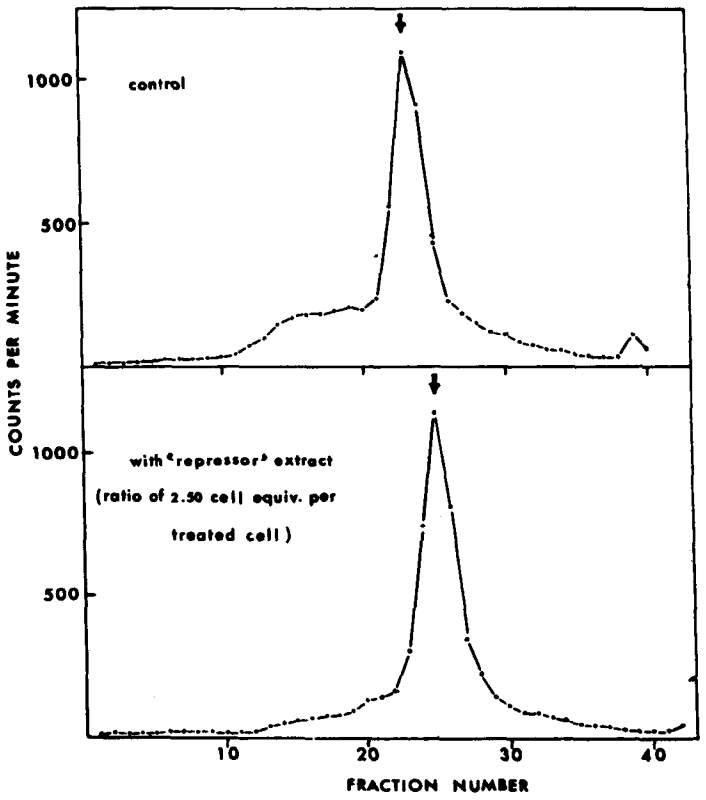

Fig. 2 - Neutral CsCl gradients of 48 hrs. viral DNA extracts (m.o.i. = 0.5 PFU/cell). Arrows indicate the position of marker SV 40 form I DNA, as determined in a separate gradient.

In both cases, treatment of infected cells with "repressor" resulted in a reproducible reduction in virion production; the average yield was approximately 50 % less than that of untreated cells.

II. Effect of "repressor" extracts on synthesis, infectivity and physical characteristics of viral DNA.

A. DNA synthesis :

Synthesis of DNA in SV 40 virus infected CV-1 cells treated with varying levels of "repressor" extract was monitored by following incorporation of ^{3}H-thymidine. At different times after infection, viral DNA extracts were prepared by the Hirt procedure (see Materials and Methods). No differences in viral or cellular DNA synthesis could be observed in "repressor" treated samples compared with untreated controls. To avoid the possibility that a putative effect on viral DNA synthesis might be masked by contamination of the extracts with cellular DNA, the Hirt extracts were sedimented in neutral CsCl gradients to have a more accurate determination of their viral DNA content. As shown in fig. 2, an extract prepared 48 hours after infection at an m.o.i. = 0.5 contained 80 % of its radioactivity in viral DNA. At a higher multiplicity (m.o.i. = 5) or at a later time during infection (72 hours), the contamination was reduced to 5-10 %. In all gradients, fractions 20-30 were taken as the viral DNA region and corrections for cellular DNA were made on this basis. The incorporation of ^{3}H-thymidine into viral and cellular DNA corrected for contamination is given in Table I. (The table does not include corrections for possible cellular DNA within the viral region). For a given multiplicity of infection and for a given time of extraction, there was no significant difference between "repressor" treated samples and controls with respect to the level of contamination by cellular DNA, even within the viral region (see figs. 2 and 3).

Since it was possible that "repressor" extracts might affect the intracellular thymidine pool size and hence the incorporation of ^{3}H-thymidine into DNA, viral DNA specific activity was determined for several samples. No significant differences were found between "repressor" treated and control samples. Thus, incorporation studies indicate that viral DNA synthesis is not measurably affected by the "repressor".

B. Viral DNA infectivity :

The specific infectivity of the viral DNA extracts is given in table I. The DNA infectivity values are normalized for the amount of DNA in each Hirt extract. The presence of "repressor" reduces the specific infectivity by approximately 50 %. In three other experiments similar to that outlined in table I, viral DNA infectivity was also reduced by approximately 50 %. Further experiments were performed at m.o.i. = 0.05 (samples taken at 24 and 48 hours after infection) and at an m.o.i. = 0.5, (sampled at 24 hours after infection); although total infectivity was reduced by 50 %, the incorporation of ^{3}H-thymidine was too low to determine accurately the effect on specific infectivity.

C. Viral DNA size and shape :

Since the observed reduction in infectivity might be attributed to altered size or shape of the viral DNA, the Hirt extracts were analyzed by centrifugation in neutral CsCl (fig. 2) and alkaline sucrose (fig. 3) sedimentation gradients. Most of the viral DNA was in closed circular form I molecules. No affect on size or shape of viral DNA was observed.

D. Effect of trypsin on infectivity of viral DNA extracts :

In order to see if the decreased infectivity of the DNA was

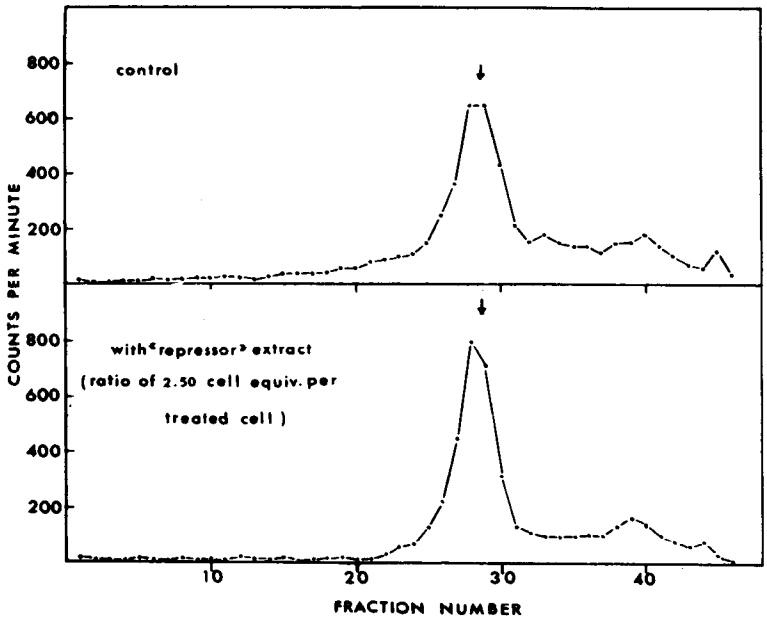

Fig. 3 - Alkaline sucrose gradients of 48 hrs. viral DNA extracts m.o.i. = 0.5 PFU/cell). Arrows indicate the position of marker SV 40 form I DNA, as determined in a separate gradient.

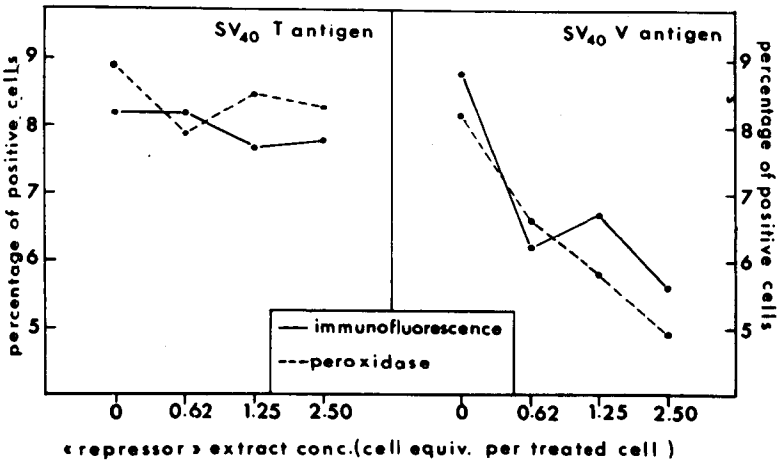

Fig. 4 - Synthesis of SV 40 tumor (T) and structural (V) antigens in CV-1 cells treated with "repressor" extracts after infection with SV 40 virus (m.o.i. = 1 PFU/cell).

due, at least in part, to the presence of protein(s) in the extracts, the sensitivity of the extracts to trypsin was determined. All samples when exposed to the trypsin inhibitor (Iniprol) alone lost infectivity. Nonetheless, it was apparent (table II) that trypsin treatment erased the difference in infectivity between untreated and "repressor" treated extracts. It is likely that this is indicative of "repressor" degradation by trypsin. However, an

TABLE I

"Repressor" extract conc. (cell equiv. per treated cell	Time of DNA extraction (Hours post inf.)	cpm/culture		PFU per culture ($\times 10^6$)	% inhibition of viral DNA specific infectivity
		Cellular DNA ($\times 10^6$)	Viral DNA ($\times 10^5$)		
Multiplicity of infection = 0.5 PFU/cell					
0	48	2.83	2.38	13.00	-
0.62		2.93	2.70	6.40	56.6
1.25		3.16	3.15	6.00	64.9
2.50		3.20	2.63	8.40	41.5
0	72	3.55	6.64	13.50	-
0.62		3.15	6.26	6.80	46.5
1.25		4.08	1.98	1.80	55.4
2.50		3.78	6.58	10.20	23.8
Multiplicity of infection = 5 PFU/cell					
0	24	2.06	0.24	0.42	-
0.62		1.74	0.28	0.35	31.1
1.25		1.94	0.44	0.31	59.8
2.50		2.22	0.20	0.39	(-)
0	48	4.46	6.68	64.00	-
0.62		3.86	6.02	15.80	73.6
1.25		4.63	7.22	21.00	69.7
2.50		4.07	6.48	43.60	29.5
0	72	4.75	12.12	78.00	-
0.62		4.73	16.47	30.00	72.8
1.25		5.55	16.23	50.00	52.5
2.50		5.12	15.20	56.00	42.8

Table I - Effect of "repressor" extracts on synthesis and infectivity of viral DNA.

alternate hypothesis, that trypsin differentially protects untreated and "repressor" treated extracts against Iniprol, is not excluded.

III. Effect of "repressor" extracts on synthesis of T and V antigens.