towards hydrolysis.

Also, it is worth noting that the fraction with the highest specific activity observed with the incorporation of C^{14} stearic acid was 1/50th of the specific activity of the fatty acids in the same sample. On the other hand, when C^{14} oleic acid was incorporated to 1,3-diglycerides, this fraction had 4 times more specific activiy of fatty acids in the same sample at 1.5 hours and 1/2 at 3 hours incubation.

In a second exchange experiment with oleic acid, specific activity of 1,3-diglycerides was about 27% at 1.5 hours and 45% at 3 hours of the specific activity of free fatty acids in the same sample. For the 1,2-diglycerides these values were 3.1% and 6.7% respectively. This indicated that oleic acid was exchanged faster than stearic acid.

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Prevention of SV_{40} Virus Tumorigenesis by Irradiated, Disrupted and Iododeoxyuridine Treated Tumor Cell Antigens.* (31851)

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Studies in these laboratories during the past several years have been directed toward the development of safe and practicable procedures for immunizing human beings against cancer utilizing animal model systems and based on the knowledge that new tumor antigens capable of inducing resistance to tumor appear in animal neoplasia. A principal aspect of the program has been the development of means for rendering tumor cells nonproliferative and for disruption of neoplastic cells without destroying the tumor antigen. The latter objective appears a requisite to tumor antigen purification with removal of normal cellular antigen components which might lead to autoimmune and other possible disorders upon application in immunization procedures.

Previous reports(1,2) by our group described the development of a model system for testing the efficacy of tumor immunization. In these studies, it was shown(2) that parenteral injection of x-irradiated SV_{40} tumor cells was highly effective in preventing SV_{40} virus tumor when the oncogenic virus was given to hamsters as newborns and the irradiated tumor vaccine was given in a single dose between day 34 and 76 following virus and prior to first appearance of virus-induced tumor. All protective activity was lost, however, when the untreated cells were disrupted by homogenization and the immunizing capacity was not preserved by prior treatment with formalin(3).

The present report describes the results of efforts to disrupt tumor cells without destroying immunizing ability and to devise additional methods for rendering tumor cells nonproliferative. In the studies, it was shown

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that cell disruption resulted in loss of immunogenicity. Further, it was shown that iododeoxyuridine (IUDR), like irradiation, was an effective method for treating tumor cells to render them nonproliferative without destroying their immunizing capability.

Materials and methods. SV_{40} virus-hamster tumor model. The detailed procedures were described earlier(1,2). By this method, random bred newborn Syrian hamsters (Lakeview Hamster Colony, Newfield, N. J.) less than 24 hours of age were injected subcutaneously into the interscapular space with 0.2 ml of the 45-54 strain of SV_{40} virus containing $10^{7.5}$ $TCID_{50}$/0.1 ml based on titration in primary grivet renal cell culture. Immunizing antigens were given in 1.0 ml amount intraperitoneally when the animals were 34 to 60 days old. Randomization of SV_{40} virus-infected hamsters for assignment to test or control groups was done at the time of weaning. Tumor detection was by weekly palpation (SV_{40} virus-induced tumors) or by autopsy (tumors arising intraperitoneally from incompletely inactivated immunizing preparations). Procedures for animal care, clinical observations, and pathologic observations were described previously(1,2). A small proportion of animals in the immunization experiments died prior to termination due either to inapparent cause or to infrequent peritoneal tumor resulting from a small number of viable tumor cells in certain of the immunizing preparations. The latter tumors were readily distinguished from the subcutaneous SV_{40} virus-induced tumors by their intraperitoneal location and were not included in the tumor counts. In the analyses in the Tables, the percentages of animals with subcutaneous tumor were adjusted according to standard life table procedures. *SV_{40} hamster tumor cells.* The virus-free F5-1 line(2,4) of SV_{40} hamster tumor was used. The cells were cultivated *in vitro* in medium 199 containing 5% heat-inactivated calf serum plus antibiotics. Cell cultures from passage 15 to 34 *in vitro* were employed. *Cesium^{137} irradiation.* Irradiation was carried out in open siliconized petri plates employing a Caesatron model E therapy unit equipped with a 10 cm $\times$ 10 cm cone and delivering 110-117 r of gamma rays per minute in air at 22 cm distance. *IUDR.* Grade A 5-iododeoxyuridine, referred to as IUDR, was obtained from California Biochemicals Co. A 10 mg/ml stock solution was prepared in Hanks' balanced salt solution (HBSS) with heating and was sterilized by filtration, while hot, through a Millipore membrane. The filtrate, cooled to 40-45°C, was diluted further in medium 199 and added to the F5-1 SV_{40} tumor cell cultures as described in the text. *Cell disruption.* The cells were suspended to desired concentration and disrupted by alternate freezing at —196°C in liquid nitrogen and slow thawing at 4°C in an ice bath or by treatment in a French pressure cell (4-3398, American Instrument Co., Silver Spring, Md.). *Vital staining.* Tests for viable tumor cells were made by adding trypan blue in a final concentration of 0.17% and observing microscopically for dye exclusion. Uptake of trypan blue stain was regarded as indicating nonviability of a cell. *Chemical determinations.* Whole or disrupted cell suspensions were fractionated over a 2-day period by the modified Schmidt-Thannhauser procedure(7,8) and the protein and ribonucleic acid (RNA) content of each preparation was determined on the appropriate cell fraction by the Lowry(5) and orcinol(6) methods, respectively. *Handling in the cold.* At no point in the process or prior to biologic or chemical determination was the temperature of the disrupted cell preparation allowed to exceed 4°C. All such preparations were fractionated and assayed chemically or injected into animals in the shortest possible time period after preparation. For chemical assay, the samples were placed in 0.5 N perchloric acid at 4°C within 1 to 2 minutes after preparation. Immunizing antigens were held in the cold and were usually injected into the animals within 1 hour of preparation.

Results. Experiment 1. The freeze-thaw procedure was employed to fragment F5-1 tumor cells under conditions which would minimize the chances for enzymatic degradation of the immunizing antigen. Aliquots of a suspension containing 1.9×10^7 per ml of washed F5-1 tumor cells in HBSS were subjected to one or three cycles of freezing and thawing as described in the section on

TABLE I. *Exp 1.* Test for Protective Efficacy of Freeze-Thaw Filt 5-1 Line SV_{40} Virus Hamster Cell Antigen Against Homologous Virus-Induced Tumor.

	Cumulative occurrence, SV_{40} virus-induced tumor			
	Test antigens		Controls	
Day following SV_{40} virus	Freeze-thaw 1×	Freeze-thaw 3×	Gamma irradiated cell antigen	Unvaccinated
1	0/32	0/32	0/32	0/32
90	0/31	0/31	0/31	0/30
120	5/30	2/31	1/31	4/30
141	13/25	8/31	1/31	13/28
148	13/22	11/28	1/30	16/28
160	18/21 (78%)*	16/27 (56%)*	1/30 (3%)*	17/26 (60%)*
Protective efficacy†	—30%‡	7%	95%	—

* Adjusted for nonspecific deaths according to standard life table procedures.
† Compared with unvaccinated control.
‡ Minus sign indicates higher tumor incidence in vaccinated than in control animals.

Materials and methods. The pH of the treated suspensions was between 7 and 8 and the period required for thawing was usually 30-35 minutes. Immediately after the last thaw, samples were taken for protein and RNA determinations and for vital staining and the remainder was diluted in cold HBSS to the equivalent of 5×10^6 cells/ml for immunization purpose. For control, a portion of the original F5-1 cell suspension containing 1.9×10^7 cells per ml was exposed to 5000 r with the Caesatron unit, diluted as above, and samples taken for protein and RNA determination and for immunization purpose. Direct microscopic examination revealed that all but a few of the cells in the freeze-thaw samples were disrupted and none of the remaining intact cells appeared to be viable based on vital staining. Groups of 32 hamsters which had received SV_{40} virus 47 days previously, as newborn, were each given 1 ml, intraperitoneally, of the freeze-thaw or irradiated cell antigens or were held without further injection.

Table I summarizes the findings in the tests for protective efficacy against SV_{40} virus tumor in the hamsters. It is seen that the freeze-thaw antigens afforded no significant protection against appearance of SV_{40} virus-induced subcutaneous tumor. By contrast, the gamma ray-irradiated preparation effected a 95% reduction in expected occurrence of tumor.

The findings in the biochemical determinations shown in Table II revealed a marked reduction in protein and comparatively less reduction in RNA in the freeze-thaw preparations. The effect was greater following 3 freeze-thaw cycles than after a single freeze-thaw step. Loss of immunizing capability accompanied apparent reduction in protein and RNA, whether causally related or not.

Experiment 2. (Tables III and IV). It was believed that the loss of immunizing potency of the tumor cell preparations in Experiment 1 above might have resulted from degradation by endogenous enzymes. An attempt was made to minimize catabolic activity by cell lysis in the chilled French pressure cell in the presence of 0.1 Molar ethylenediamine-tetraacetic acid (EDTA) at pH 8.5. The amount of chelating agent was greatly in excess of the amount necessary to bind the cations present in the preparations.

Conduct of the experiment was the same as Exp. 1 above except for preparation of the antigens. Washed F5-1 tumor cells at a concentration of 1.2×10^7 viable cells/ml served as starting material. The pressure cell

TABLE II. *Exp 1.* Effect of Rapid Freeze-Thaw on the Protein and RNA Content of Filt 5-1 Line SV_{40} Hamster Tumor Cells.

	Content ($\mu g/5 \times 10^6$ cells)	
Treatment	Protein	RNA
Test:		
Freeze-thaw 1 time	710	446
" " 3 times	248	336
Control:		
Gamma irrad. (5000 μg)	1000	573
Untreated	900	613

material was prepared by treating 25 ml of cell concentrate in EDTA-HBSS in the chilled French pressure cell for 60 seconds at 10,000 psi. followed by extrusion and dilution into cold 0.1 M EDTA in HBSS. The irradiated control tumor cell suspensions were exposed to 3500 r in the Caesatron unit. Pressure cell disrupted antigen and the gamma irradiated antigen were prepared in alum adjuvant. By the procedure used, 3.7 ml of sterile 10% sodium phosphate solution was added slowly to 49.7 ml of cell suspension in EDTA (disodium salt). The pH shifted to 6.5. Finally, 5.24 ml of sterile 10% $KA1SO_4$ was added with stirring, keeping the pH at 6.2 to 6.5 by addition of 1 N NaOH. Alum suspension alone, for control purpose, was prepared substituting EDTA-HBSS for cell suspension. The final antigens contained the equivalent of 3.6×10^6 original tumor cells.

TABLE III. *Exp 2.* Test for Protective Efficacy of Pressure Cell Disrupted and Alum-Adsorbed Filt 5-1 Line SV_{40} Hamster Cell Antigen Against Homologous Virus-Induced Tumor.

Day following SV_{40} virus	Cumulative occurrence, SV_{40} virus-induced tumor					
	Test antigens		Controls			
	Pressure cell only	Pressure cell—alum ads.	Gamma irrad.	Gamma irrad.—alum ads.	Alum alone	Unvaccinated
1	0/32	0/31	0/31	0/31	0/30	0/28
114	5/26	6/30	1/26	2/29	2/29	2/25
142	12/24	19/28	1/19	13/28	13/29	13/24
161	13/23	20/28	2/18	15/26	17/29	15/24
181	19/21	23/27	2/10	15/26	21/29	18/22
292	20/20 (95%)*	27/27 (100%)*	2/5 (40%)*	17/24 (63%)*	24/26 (85%)*	21/21 (97%)*
Protective efficacy†	2%	—3%	59%	35%	—	—

* Adjusted for nonspecific deaths according to standard life table procedures.
† Compared with unvaccinated control.

The findings shown in Table III revealed that the pressure cell disrupted antigen was noneffective in preventing SV_{40} virus tumor and that the potency was not improved by added alum. The degree of suppression of tumor by irradiated cell antigen was less than ordinarily obtained. The latter finding was more apparent than real and was due to incomplete destruction of cell viability which resulted in 21 vaccine-induced deaths from peritoneal tumors. Treatment of the irradiated cells with alum suppressed the formation of peritoneal tumors but also markedly reduced the immunizing efficacy. As seen in Table IV, the pressure cell treatment employed resulted in marked destruction of both protein and RNA. The RNA content was preserved by addition of alum but there was only partial protection of the protein.

Experiment 3. The possibility was considered that irradiation might be required to render the tumor cells antigenic or that such treatment might reduce the extent of antigenic degradation brought about by cell disruption. An experiment was undertaken,

TABLE IV. *Exp 2.* Effect of Pressure Cell Disruption on the Protein and RNA Content of Filt 5-1 Line SV_{40} Hamster Tumor Cells.

Treatment	Content ($\mu g/3 \times 10^6$ cells)	
	Protein	RNA
Test:		
Pressure cell only	92	360
Pressure cell—alum ads.	160	752
Control:		
Gamma irrad.	202	730
Gamma irrad.—alum ads.	204	710
Untreated	218	750

TABLE V. *Exp 3.* Test for Protective Efficacy of Gamma Irradiated-Pressure Cell Disrupted Filt 5-1 Line SV_{40} Hamster Cell Antigen Against Homologous Virus-Induced Tumor.

	Cumulative occurrence, SV_{40} virus-induced tumor				
	Test antigens			Controls	
Day following SV_{40} virus	Pressure cell only	Gamma irrad.—pressure cell	Gamma irrad. only	Buffer solution	Unvaccinated
1	0/24	0/26	0/31	0/10	0/32
99	1/21	0/24	0/31	1/9	0/32
112	1/21	2/21	0/31	1/9	1/32
127	4/21	2/21	0/31	2/9	4/32
148	7/20	11/21	0/31	5/9	9/29
169	11/19	11/21	0/31	5/9	16/27
190	15/17	16/19	1/30	5/9	18/26
240	16/17 (90%)*	16/19 (79%)*	2/30 (7%)*	7/9 (78%)*	20/23 (75%)*
Protective efficacy†	—20%‡	—5%‡	91%	—	—

* Adjusted for nonspecific deaths according to standard life table procedures.
† Based on comparison with unvaccinated control.
‡ Minus sign indicates greater tumor incidence in vaccinated than in control animals.

therefore, in which the F5-1 cell suspension was irradiated prior to cell disruption. An F5-1 tumor cell suspension containing 1.2×10^7 cells/ml was prepared in a calcium- and magnesium-free (CMF) buffered salt solution containing 2 mg of glucose/ml.

A portion of the cells was exposed to 4000 r with the Caesatron unit. Aliquots of the irradiated and nonirradiated cell suspensions were then treated with EDTA to give a 0.1 M solution and the cold suspensions were passed twice through the chilled (4°C) French pressure cell at 8000 psi. They were then diluted to the equivalent of 4.8×10^6 cells per ml and no viable cells were detectable by vital staining. Samples were taken immediately for chemical fractionation and assay and and 1 ml amounts of the preparations were injected intraperitoneally into hamsters which had received SV_{40} virus 42 days earlier. Table V shows that the irradiated cell preparation was highly effective in preventing virus tumor. Pressure cell disrupted cells, whether previously irradiated or not, were without effect. As seen in Table VI, there was marked reduction in protein and in RNA in the pressure cell treated materials whether irradiated prior to disruption or not.

Experiment 4. Suppression of F5-1 cell proliferation by IUDR. Studies were undertaken to ascertain whether the proliferative ability of F5-1 cells could be destroyed while retaining immunizing potency by incorporation of thymidine analogue IUDR. In an initial experiment (Fig. 1) washed F5-1 cells were planted in replicate cultures in tubes in the presence of growth medium containing graded amounts of IUDR and incubated at 37°C. Two of the replicate tubes from each drug concentration were sampled daily for viable cell counts for 7 days. Additionally, 2 tubes were washed free of IUDR on day 5, counted, replanted in IUDR-free medium and counts of viable cells were made after 1 and 2 days further incubation. It is seen in the Figure that suppression of cell proliferation was directly proportional to the concentration of IUDR employed. Total suppression of cell proliferation was achieved at the 500 and 700 μg levels following the third day of incubation at which time no viable cells were detectable on vital staining. Cells which were treated with lesser concentration of IUDR also stopped proliferating following the third day of incubation but viable cells could be

TABLE VI. *Exp 3.* Effect of Gamma Irradiation Followed by Pressure Cell Disruption on the Protein and RNA Content of Filt 5-1 Line SV_{40} Hamster Tumor Cells.

	Content ($\mu g/5 \times 10^6$ cells)	
Treatment	Protein	RNA
Test:		
Pressure cell only	205	898
Gamma irrad.—pressure cell	183	720
Gamma irrad. only	507	1270
Control:		
Untreated	570	1500

counted through the seventh day. Though viable, these cells were incapable of proliferating *in vitro* following removal of the IUDR and replanting on day 5. By contrast, untreated control cells continued proliferation through day 5 and there was further growth on replanting.

In a similar test, the IUDR was added to

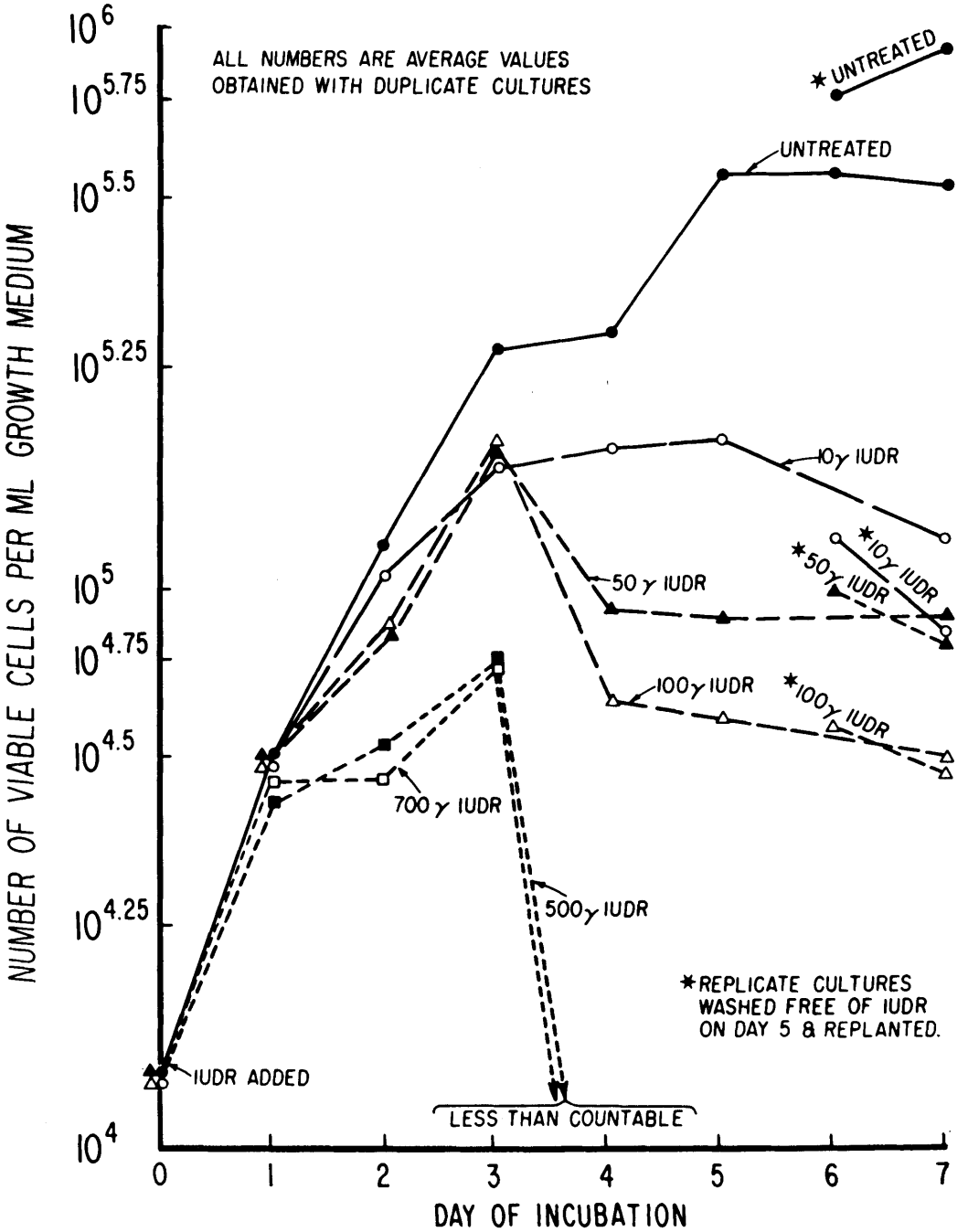

FIG. 1. Influence of added iododeoxyuridine on the proliferation, *in vitro*, of filt 5-1 SV_{40} virus-induced hamster tumor cells. IUDR was added *at time of* planting cultures.

the cultures 24 hours after planting with the results shown in Fig. 2. A similar pattern of suppression of cell proliferation was noted except for a less dramatic reduction in viable cell count at the high drug concentrations, 500 and 700 μg. As in Exp. 1, the cells which were treated for 5 days with IUDR failed to proliferate upon removal of drug and replanting.

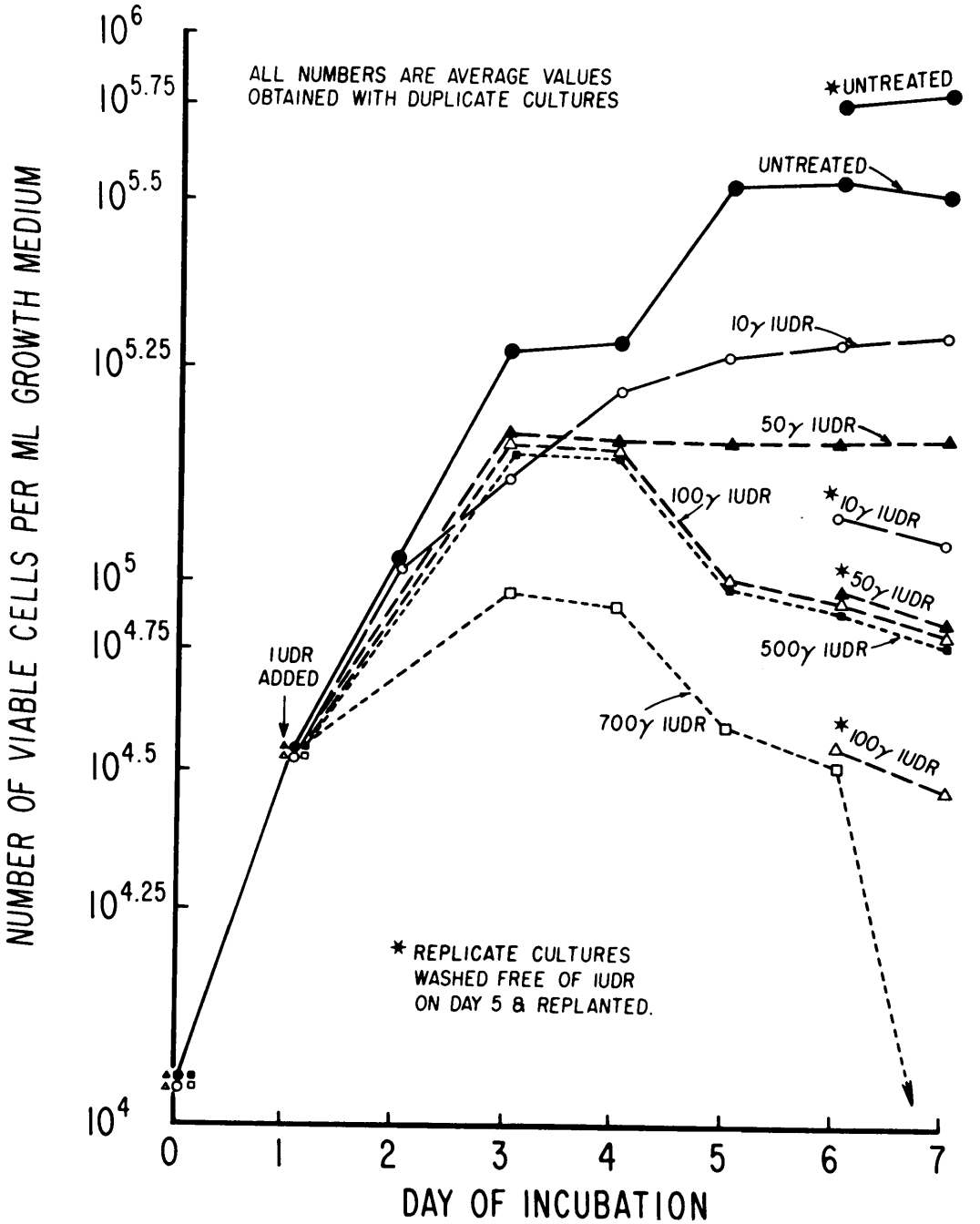

FIG. 2. Influence of added iododeoxyuridine on the proliferation, *in vitro,* of filt 5-1 SV_{40} virus-induced hamster tumor cells. IUDR was added *24 hours after* planting of cultures.

TABLE VII. *Exp 5.* Protective Efficacy of Iododeoxyuridine (IUDR)-Treated Filt 5-1 Line SV_{40} Hamster Cell Antigen Against Homologous Virus-Induced Tumor.

Day following SV_{40} virus	Cumulative occurrence, SV_{40} virus-induced tumor			
	Test antigen	Controls		
	IUDR-treated	Gamma irrad.	Hanks' BSS	Unvaccinated
1	0/32	0/32	0/8	0/32
131	0/32	0/31	0/8	4/32
158	2/31	0/31	6/8	19/32
166	2/31	0/31	6/8	21/32
173	3/30	0/30	8/8	22/32
186	3/30	0/30	8/8	24/32
199	3/26 (10%)*	0/24 (0%)*	8/8 (100%)*	29/31 (92%)*
Protective efficacy†	90%	100%	—	—

* Adjusted for nonspecific deaths according to standard life table procedures.
† Based on comparison with unvaccinated control group.

The destruction by IUDR of replicative capacity of F5-1 cells *in vitro* was shown more fully in a test in which 100 γ of IUDR/ml was added to replicate cultures in Blake bottles 24 hours after seeding. In one series (A), bottles were harvested daily and viable cell counts were made. In a second series (B), the cells from individual bottles were harvested on the second through seventh days of incubation, washed free of IUDR and replanted in growth medium without drug. Untreated cell cultures were included for control purpose. Counts for viable cells in individual bottles were made 6 or 7 days thereafter. It is seen in Fig. 3 that proliferation of F5-1 cells (Series A) was stopped within 24 hours following addition of drug. This was in contrast to the experiment summarized in Fig. 2 in which an approximate $10^{0.75}$ log increase in cells in culture tubes occurred after addition of drug. The cultures (Series B) which were washed free of IUDR 24 hours or more following addition of drug and replanted all failed to proliferate on subpassage in drug-free growth medium.

Experiment 5. Immunizing capacity of IUDR treated F5-1 cells. F5-1 cells in culture were treated with 100 γ/ml of IUDR 24 hours after planting and incubated for 4 additional days. The cells were suspended in HBSS, washed, adjusted to contain 4.5×10^6 viable cells per ml, and inoculated intraperitoneally in 1 ml amount into hamsters which had received SV_{40} virus 34 days earlier as newborns. Control materials employed in the immunization experiments consisted of non-IUDR treated cells of similar concentration exposed to 4000 r in the Caesatron unit or irradiated HBSS. A portion of animals which were not injected further served as additional controls. It is seen in Table VII and graphically in Fig. 4 that the irradiated cell antigen control effected 100% suppression of virus-induced tumor compared with the nonimmunized controls. The IUDR-treated cells effected a 90% suppression of tumor occurrence and were nearly as effective as irradiated cells as immunizing antigen.

Discussion. The hamster-SV_{40} tumor model developed in our laboratories(1,2) has provided an excellent system for exploring the possibilities for prophylaxis of hypothetical neoplasia of man caused by viruses. Using this model, tumor is prevented in hamsters given oncogenic virus when newborn and given homologous immunizing tumor antigen at a later time period but prior to first appearance of virus-induced tumor. In the previous studies(2), x-irradiated hamster tumor cell suspensions were shown to be highly effective as immunizing antigens for preventing tumor. The present studies with tumor cell preparations rendered nontumorigenic by exposure to gamma rays from a $Cesium^{137}$ source have abundantly confirmed the high level efficacy of such antigen for preventing the appearance of virus-induced tumor.

The findings in the present report relating to IUDR confirm the reports by others(9-12) that tumor cells may be rendered nonreplicative by the analogue, presumably by incorporation into the DNA of the cell. It is

of considerable interest that tumor cells rendered incapable of replication by IUDR displayed a high level of effectiveness for preventing virus-induced tumor which ap-

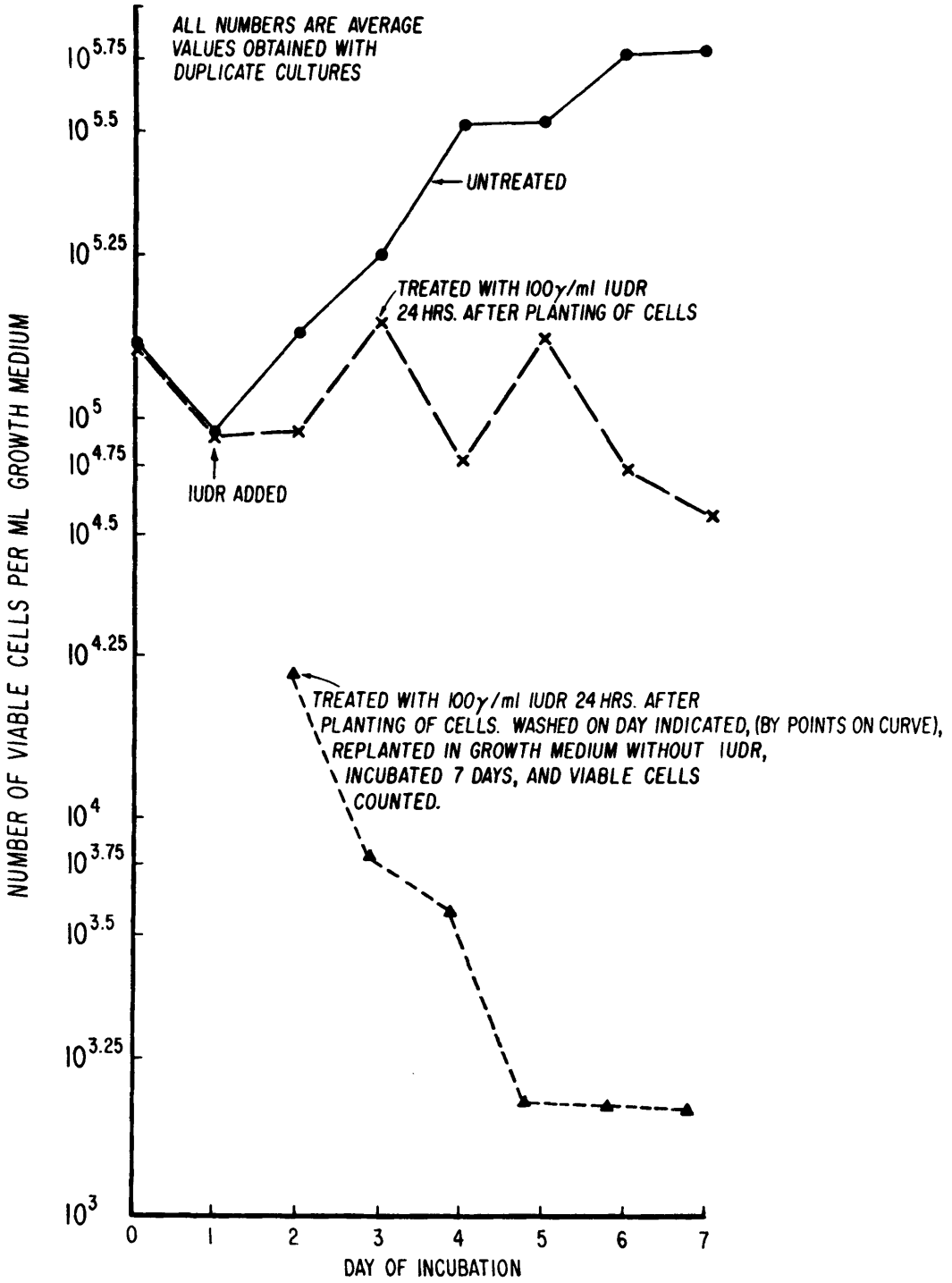

FIG. 3. Establishment of destruction of *in vitro* replicative capability of filt 5-1 SV_{40} virus-induced hamster tumor cells by addition of 100γ/ml of iododeoxyuridine (IUDR).

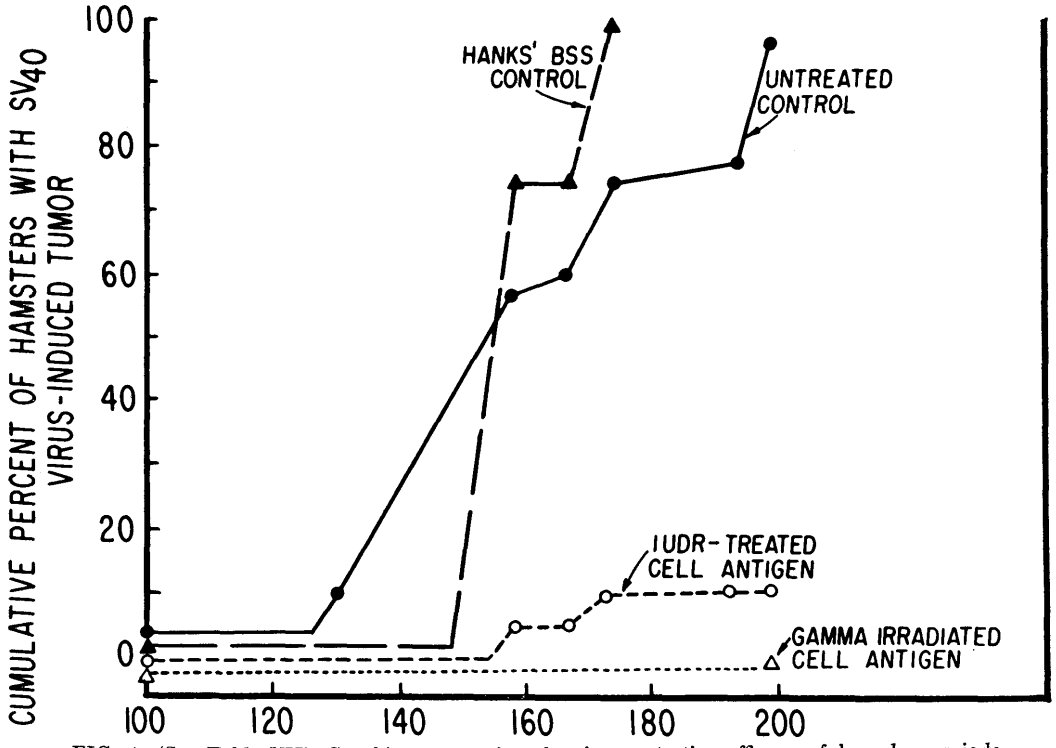

FIG. 4. (See Table VII) Graphic presentation showing protective efficacy of homologous iododeoxyuridine (IUDR) and gamma-irradiated cell antigens against SV_{40} virus-induced tumor. (All values adjusted to life expectancy tables.)

proximated that of irradiated cell preparations. Such effect was not due to residual IUDR since it was found in other experiments not reported in the text that inoculation of 1 ml of solution containing 100 mg IUDR into newborn hamsters infected with SV_{40} virus did not suppress tumor appearance. The IUDR method offers an alternative procedure for preparing nonreplicating tumor antigen and offers another possibility for use in preparing antigen for treating autochthonous tumor in man.

The unique antigen which is present in SV_{40} virus-induced tumor of animals and which affords resistance to tumor transplantation is specific to virus species and not to host cell species(13). Such antigen might be of utility for preventing hypothetical virus tumor in man or for treating metastatic tumor in human subjects provided it can be sufficiently freed from extraneous antigens which might lead to hypersensitization, autoimmune type reactions, or immunologic enhancement of tumor growth.

All efforts in our laboratory to date to disrupt tumor cells preparatory to extracting tumor antigen or otherwise remaining extraneous antigens has resulted in destruction of immunizing activity as measured in the hamster-SV_{40} tumor model. This is in contrast to histocompatability antigens which may be sufficiently antigenic and stable to permit induction of transplantation resistance using disrupted cell fractions for immunization purpose(14,15).

The findings in the present studies provide a possible explanation for loss of antigen following cell disruption based on release of intracellular enzymes by freeze-thaw or by treatment in the French pressure cell which appear to cause destruction of cellular protein and RNA. Alternatively, the loss of immunizing potency by cell disruption might be due more to prevention of further synthesis of tumor antigens by gamma-irradiated or IUDR-treated cells than to destruction of existing antigen.

Studies along these lines are continuing

with special emphasis on the development of procedures for inactivating tumor cell enzymes without destruction of tumor antigen.

Summary. Hamster SV_{40} tumor cells rendered nonproliferative by exposure to gamma rays or by propagation in the presence of iododeoxyuridine were highly effective when used as immunizing antigens for preventing the appearance of tumors in hamsters which had received SV_{40} virus when newborn. Only a single injection of immunizing antigen was employed. Disruption of tumor cell preparations by freeze-thaw or by treatment in a French pressure cell for purpose of fractionation resulted in total or near total loss of immunizing capability, even when incorporated into alum adjuvant. Possible mechanisms for loss of potency are discussed.

ADDENDUM: Since the time the present manuscript was in press, it was found in further experiments employing the same techniques that the loss of RNA and protein following cell disruption was considerably less than noted above.

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Dependency of Filtration and P-Aminohippurate (PAH) Secretion on Na Reabsorption in the Obstructed Dog Kidney.* (31852)

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Creatinine and p-aminohippurate (PAH) accumulate in renal tissue during ureteral obstruction in oliguria and in mannitol diuresis (1). Accumulation of creatinine was not significantly different from inulin in experiments in which both substances were used. It was concluded that accumulation of creatinine could be attributed to persistent filtration during the period of obstruction. Although reabsorption of fluid from the lumen during stop-flow was suggested as the cause of persistent filtration, the relationship between these two variables was not established.

A hypothesis has been devised to relate so-

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