RADIOBIOLOGICAL AND BIOCHEMICAL INVESTIGATIONS OF POLYOMA VIRUS - CELL INTERACTIONS

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TO

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ABSTRACT

The cytolytic interaction of Polyoma virus with mouse embryo cells has been studied by radiobiological methods known to distinguish temperate from virulent bacteriophage. No evidence for "temperate" properties of Polyoma was found. During the course of these studies, it was observed that the curve of inactivation of Polyoma virus by ultraviolet light had two components - a more sensitive one at low doses, and a less sensitive one at higher doses. Virus which survives a low dose has an eclipse period similar to that of unirradiated virus, while virus surviving higher doses shows a significantly longer eclipse period. If Puromycin is present during the early part of the eclipse period, the survival curve becomes a single exponential with the sensitivity of the less sensitive component. These results suggest a repair mechanism in mouse cells which operates more effectively if virus development is delayed.

A comparison of the rates of inactivation of the cytolytic and transforming abilities of Polyoma by ultraviolet light, X-rays, nitrous acid treatment, or the decay of incorporated P^{32}, showed that the transforming ability has a target size roughly 60% of that of the plaque-forming ability. It is thus concluded that only a fraction of the viral genes are necessary for causing transformation.

The appearance of virus-specific RNA in productively infected mouse kidney cells has been followed by means of hybridization between pulse-labelled RNA from the infected cells and the purified virus DNA. The results show a sharp increase in the amount of virus-specific RNA around the time of virus DNA synthesis. The presence of a small amount of virus-specific RNA in virus-free transformed cells has also been shown. This result offers strong evidence for the persistence of at least part of the viral genome in transformed cells.

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GENERAL INTRODUCTION

The phenomenon of virus-induced neoplastic transformation of animal cells in culture (referred to hereafter simply as transformation) has become the object of an increased amount of attention and investigation in recent years. The term "transformation" is broadly used to describe the acquisition of certain heritable characteristics referring both to cellular morphology and, more frequently, to patterns of cell growth and interaction - specifically, multilayered and criss-cross growth pattern, and loss of contact inhibition[1]. Cells transformed in vitro by viruses have been shown to give rise to tumors when injected into animals, and primary virus-induced tumors transplanted into culture show growth properties similar to those of transformed cells. On the basis of such observations, transformation is generally regarded as the in vitro counterpart of tumor-induction.

Significant progress in understanding this phenomenon has been made during the last few years, due in part to improvements in techniques for studying virus-cell interactions in tissue culture, and also to rapidly expanding knowledge in fundamentally related fields, such as biochemistry, physiological phage genetics, etc. Studies of two viruses in particular have been fruitful in defining the possible roles of virus in transformation. One is the Rous Sarcoma Virus (RSV), representative of the group of RNA-containing Avian leukosis viruses[2], and the other is polyoma virus (Py), a member of a group of small DNA-containing Mammalian tumor viruses which also includes rabbit papilloma

virus, human warts virus, and SV-40 virus. A brief description and summary of the important findings with these two viruses will be given, followed by an explanation of the plan of the thesis and the purposes behind the experiments.

A. Results with Rous Sarcoma Virus

Particles of RSV are roughly spherical, about 100 mμ in diameter, and contain protein and lipid in addition to RNA[3,4]. Release of virus from infected fowl cells in culture occurs continuously and without associated cell death[5]. When infected at high multiplicity, most or all of the cells become transformed[6] and release virus; however, at low multiplicities of infection, non-virus-producing transformed cells can be isolated. The production of RSV in transformed cells is linked to the presence of "helper" virus normally present in RSV stocks. The helper virus can be any of several viruses in the Avian leukosis group. Non-virus-producing transformed cells, grown for many cell generations, can be made to release RSV by reinfection with one of the helper viruses. The antigenic specificity of the RSV thus produced is the same as that of the helper virus[7,8,9,10]. These findings have shown that RSV is a defective virus, altered in some late function involved in the synthesis of the protein coat, and that the defective genome persists in the transformed cells. Furthermore, a direct role of the RSV genome in determining properties of the transformed cells is shown by the fact that different virus mutants induce different morphological types of transformed cells[11].

B. Results with Polyoma Virus

Py is a small icosahedral virus, approximately 43 mμ in diameter[12], and consists of protein and a double-stranded circular DNA of molecular weight 3 x 10^6 [13,14]. The virus is capable of inducing a wide variety of tumors in rodents[15]. Both the cytolytic interaction with embryonic mouse cells[16,17] and the non-cytolytic (transformation) interaction with hamster cells[18,19] can be obtained with the viral DNA alone[20,21]. The effeciency of the virus for transformation is low, roughly 10^{-4} of that for plaque formation. In addition, a maximum of only a few per cent of infected cells become transformed after a single exposure to a high concentration of virus[22].

Efforts to induce the development of infectious virus from transformed cells by a variety of treatments known to induce the development of prophage have given only negative results[23,24]. Attempts to detect viral nucleic acid by hybridization techniques have so far not been entirely conclusive[25,26]. This point will be discussed further in Part III of this thesis.

Presumptive evidence for the presence of at least a part of the viral genome in transformed cells has been obtained by the demonstration of new antigens - a specific tumor or transplantation antigen, presumably on the cell surface, which is responsible for the rejection of transplanted cells by virus-immunized animals[27,28], and an apparently virus-specific complement-fixing antigen[29]. These two antigens are distinct and unrelated to the capsid protein of the virus. There is no conclusive evidence for these antigens being specified by viral

genes - with the possible exception of the finding of a strain of Py which causes the induction of a tumor antigen with altered specificity[30].

Attempts to "rescue" viral genetic markers from transformed cells by superinfection have been reported. Although negative results were obtained using plaque-size mutations as markers[31], positive results were obtained with the strains of virus differing in specificity of the transplantation antigen induced[32].

Evidence concerning the specific nature of viral functions is limited; however, two observations should be noted: 1) Stimulation of the synthesis of enzymes of the DNA pathway together with a de-repression of cellular DNA synthesis during the cytolytic interaction of Py with mouse kidney cells has been reported[33], and 2) the isolation and characterization of a temperature-sensitive mutant of Py has been reported[34]; although the precise nature of the sensitive step is unknown, it appears to be necessary for the initiation, but not the maintenance, of transformation.

In conclusion, the following well-established facts must be taken into account in the formulation of any model of transformation by Py:

1. The amount of genetic material carried by the virus is small, perhaps 5 to 7 genes.
2. No infectious virus, or infectious DNA, or capsid protein can be found or induced in transformed cells.
3. New virus-related antigens are found associated with transformed cells.

C. Outline of the thesis

The experiments described in this thesis, on both the cytolytic and transforming interactions of Py in tissue culture, encompass two basic experimental approaches - radiobiological and biochemical. The results are presented in three Parts, organized around the experimental methods - the first two radiobiological, and the last biochemical. In the fourth part a summary of results is given along with general conclusions concerning the possible significance of some of the results in clarifying the role of Py in transformation. A brief introduction and statement of purpose for the first three Parts follow.

1. Part I is a radiobiological study of the cytolytic interaction of Py with mouse embryo cells. The experiments were suggested by the analogy between transformed cells and lysogenic bacteria. Although this analogy seemed unlikely from the negative results of induction experiments[23,24], the possibility remained open that Py could establish itself in the transformed cell as a non-inducible or defective provirus. In this case, little would be gained by continuing with similar experiments on the transformed cells themselves. However, the hypothesis could still be examined, although indirectly, by studying the cytolytic interaction, since radiobiological methods have been able to differentiate temperate from virulent bacteriophage in their lytic interactions[35]. A study of Py's cytolytic interaction was therefore undertaken as part of the more general question of whether tumor viruses could be distinguished in their cytolytic interaction by properties similar to those of temperate phage.

2. Part II is a study of the inactivations of the cytolytic and transforming abilities of Py by ultraviolet light, X-rays, nitrous acid and decay of incorporated P^{32}. Since the rate of inactivation of viruses by radiation (and other agents) can be correlated under certain circumstances with the amount of genetic material of the virus[36], it seemed reasonable to ask whether the participation or "genetic contribution" of Py in the two interactions could be distinguished experimentally in this way. The establishment of an improved quantitative assay for transformation, based on the enhanced ability of transformed cell to grow in agar compared to that of normal cells[37], made it feasible to answer this question.

3. Part III describes the results of experiments designed to test for virus-specific RNA in both productively infected and transformed cells using nucleic acid hybridization. Various modifications of this general technique have already been used successfully to study homology between phage λ and E. coli[38] and the synthesis of phage-specific RNA in infected bacteria[39]. Several problems with regard to Py seem potentially open to examination by this approach - e.g., the question of the presence of viral DNA or RNA in transformed cells, the specificity of transcription of viral DNA during virus development, and comparisons by way of "competition" experiments between cytolytic and possible viral transforming RNA. In the experiments reported in Part III, the examination of some of the initial questions is begun.

References

1. Aberchrombie, M., Cold Spring Harbour Symposium in Quantitative Biology XXVII, 427 (1963).
2. Beard, J.W. Subcellular Particles in the Neoplastic Process (F.P. Rhodes, ed.) 473, (1957) New York Academy of Sciences, N.Y.
3. Crawford, L.V., Virology 12, 143 (1960).
4. Crawford, L.V., and Crawford, E.M., Virology 13, 227 (1961).
5. Temin, H.M., and Rubin, H., Virology 8, 209 (1959).
6. Rubin, H., Virology 10, 29 (1960).
7. Hanafusa, H., Hanafusa, T., and Rubin, H., Proc. Nat. Acad. Sci. 49, 572 (1963).
8. Temin, H., Cold Spring Harbor Symposium Quantitative Biology XXVII, 407 (1962).
9. Hanafusa, H., Hanafusa, T., and Rubin, H., Proc. Nat. Acad. Sci. 51, 41 (1964).
10. Hanafusa, H., Hanafusa, T., and Rubin, H., Virology 22, 591 (1964).
11. Temin, H.M., Virology 10, 182 (1960).
12. Klug, A., J. Mol. Biol. 11, 424 (1965).
13. Weil, R., and Vinograd, J. Proc. Nat. Acad. Sci. 50, 730 (1963).
14. Dulbecco, R., and Vogt, M., Proc. Nat. Acad. Sci. 50, 236 (1963).
15. Stewart, S.E., Advances in Virus Research 7, 61 (1960).
16. Dulbecco, R., and Freeman, G., Virology 8, 396 (1959).
17. Winocour, E., and Sachs, L., Virology 8, 397 (1959).
18. Vogt, M., and Dulbecco, R., Proc Nat. Acad. Sci 46, 365 (1960).

19. Soker, M.G.P. and Macpherson, I.A., Virology 14, 359 (1961).

20. Weil, R., Virology 14, 46 (1961).

21. Crawford, L., Dulbecco, R., Fried, M., Montagnier, L, and Stoker, M, Proc. Nat. Acad. Sci. 52, 148 (1964).

22. Stoker, M.G.P., and Abel, P., Cold Spring Harbor Symposium in Quantitative Biology XXVII 375 (1962).

23. Habel, K., and Silverberg, R.J., Virology 12, 463 (1960).

24. Vogt, M., and Dulbecco, R., Virology 16, 41 (1962).

25. Winocour, E., Virology 25, 288 (1965).

26. Axelrod, P., Bolten, E.T., and Habel, K., Science 146, 1466 (1964).

27. Sjogren, H.O., Hallstrem, I., and Klein, G., Cancer Res. 21, 329 (1961).

28. Habel, K., Proc. Soc. Exptl. Biol. Med. 106, 722 (1961).

29. Habel, K., Virology 25, 55 (1965).

30. Hare, J.D., Proc. Soc. Exptl. Biol. Med. 115, 805 (1964).

31. Dulbecco, R., and Vogt, M., The Molecular Basis of Neoplasia 475 (1962) University of Texas Press.

32. Ting, Virology 24, 227 (1964).

33. Dulbecco, R., Hartwell, L., and Vogt, M., Proc. Nat. Acad. Sci. 53, 403 (1965).

34. Fried, M., Thesis, California Institute of Technology (1965).

35. Stent, G. Advances in Virus Research 5, 95 (1958).

36. Lea, D.E., Actions of Radiations on Living Cells Cambridge University Press, second ed., ch. IV, (1955).

37. Macpherson, L.A., and Montagnier, L., Virology 23, 291 (1964).

38. Green, M.H., Proc. Nat. Acad. Sci. 50, 1177 (1963).

39. Hall, B.D., Nygaard, A.P., and Green, M.H., J. Mol. Biol. 9, 143 (1964).

PART I

A Radiobiological Study of the Polyoma Virus-Mouse Embryo Cell Interaction[1,2]

Running Title: Radiobiology of Polyoma

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SUMMARY

The curve of inactivation of Polyoma Virus by ultraviolet light shows two components--a more sensitive one at low doses and a less sensitive one at higher doses. Virus which survives a low dose, corresponding to the more sensitive component, has an eclipse period nearly the same as that of unirradiated virus, while virus surviving a higher dose, corresponding to the less sensitive component, shows a significantly longer eclipse period. If Puromycin is present during the early phase of virus development, the survival curve becomes a single exponential with the sensitivity of the less sensitive component. These results suggest a host cell repair mechanism in mouse embryo cells which operates more effectively when virus development is delayed.

Experiments were conducted to determine if Polyoma exhibits radiobiological properties analogous to those previously described for

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2. Abbreviations used: Py - Polyoma Virus, HSV - Herpes Simplex Virus, ME - mouse embryo, UV - ultraviolet light, HCR - host cell repair.

temperate bacteriophage. For comparison with a virulent virus, parallel experiments were carried out with Herpes Simplex Virus. The results failed to reveal "temperate" properties of Polyoma.

INTRODUCTION

The radiobiological behavior of temperate and virulent bacteriophage has been studied extensively, and the results summarized and discussed by Stent (18). In brief, temperate phage such as λ and P22 are characterized by a low intrinsic UV sensitivity (i.e., the ratio of the rate of inactivation to the DNA content) and a high sensitivity of the capacity of the host cell to support virus growth, while the opposite is true for virulent phage such as T2 and T4. Although the reasons are not completely understood, the properties of phage-bacterial systems revealed by these studies may be a reflection of the basic difference between the two kinds of viruses--namely, the ability of temperate phage to enter into a stable relationship with its host and the absence of that ability in virulent phage.

Similar studies have been carried out in the case of RNA-containing animal viruses--Rous Sarcoma Virus (11,12) which produces tumors, and measles virus (1) which does not produce tumors. Both viruses exhibited some "temperate" properties when compared with another virus studied in the same cell system--Newcastle Disease Virus in the former case, and poliomyelitis virus in the latter. The relevance of the temperate phage analogy to the RNA viruses is, however, not clear. In the present investigation, this kind of analysis is extended to DNA-containing viruses. Herpes Simplex Virus (HSV) is used as a comparison for

Polyoma (Py). Both viruses have a lytic interaction with mouse embryo (ME) fibroblasts. Py is synthesized in the nucleus, as is the nucleocapsid of HSV. While Py is known to cause tumors and has a non-cytocidal interaction with certain cells in vitro (19,21), HSV is known only as a cytopathic virus. The comparative study of these two viruses thus affords an opportunity to determine whether the radiobiological behavior of a known oncogenic virus in its cytolytic interaction might reflect in some way its oncogenic potential.

MATERIALS AND METHODS

Virus preparations. Stocks of either the large or small plaque variety of Py were prepared from baby mouse kidney cultures according to the method of Winocour (25). Titers ranged from 4×10^8 - 1×10^9 pfu/ml. When necessary, further concentration and purification were obtained by centrifugation and CsCl density gradient fractionation (25). The MP-strain of HSV was obtained through the courtesy of Dr. Bernard Roizman of the Department of Microbiology, University of Chicago. The virus, previously grown on Hep-2 cells, was adapted to grow on mouse embryo cultures by repeated passage. Crude lysates containing 5×10^4 - 5×10^6 pfu/ml were obtained by sonication of infected cells and elimination of cell debris by low speed centrifugation. Virus stocks were stored at -70°C.

Virus assays. Plaque assays for Py were carried out on mouse embryo secondary monolayers as previously described (5). HSV assays were performed on the same cells under similar conditions. Virus in

0.1 ml volume was adsorbed to the monolayers at 37°C for 2-1/2 hours. The cultures were then overlaid with 7 ml of reinforced Eagle's medium containing 0.9% agar and 5% calf serum. Plaques were counted on the 3rd or 4th day.

Infectivity assay of Py DNA. Infective DNA of Polyoma, prepared by phenol extraction of density gradient-purified virus, was assayed according to the procedure of Weil (23).

Irradiations.

A. Ultraviolet. A 15-watt "germicidal" lamp (Westinghouse G15T8) housed in an open-end rectangular metal box was used as a source of UV. Most of the UV energy is emitted as the 2537 Å line of Mercury. A constant dose rate was assured by feeding the lamp through a "Sola" voltage stabilizer and allowing the lamp to warm up for 15 minutes before use. The effective output of the lamp was calibrated using the inactivation of bacteriophage T2 as a standard. T2 lethal doses given below refer to the survival in the absence of photoreactivation.

Virus was diluted in a Tris-salt buffer containing 0.05% calf serum. 0.2 ml of virus suspension was placed on a watch glass at a distance of 10 cm from the rim of the lamp housing. The optical density and sample thickness were such as to allow a transmittance at 260 mμ equal to or greater than 96%. Irradiations were carried out for various times as indicated. The lethal dose (37% survival) for T2 under these conditions was 0.9 seconds.

Cells were irradiated as monolayers in plastic petri dishes 6 cm in diameter containing 2 cc of Tris-salt buffer with 0.05%

calf serum. The distance was 30 cm from the rim; the T2 lethal dose was 2 seconds.

B. X-irradiation. A 250 KV Westinghouse Industrial X-ray Unit with the beam filtered through 1 mm of Aluminum was used in all experiments. For irradiation of virus, the unit was operated at 220 KV and 12 milliamps. The virus was suspended in Tris-salt buffer with 5% calf serum, which afforded ~~complete~~ a high degree of protection against indirect effects. The dose rate under these conditions was estimated at 3, 250 roentgens per minute by T2 inactivation, using a lethal dose of 40,000 roentgens (22).

Cells were irradiated either as monolayers or in suspension (in Tris-salt buffer, 5% calf serum) with 220 KV X-rays at dose rates ranging from 100-1000 roentgens per minute. The rates were measured prior to exposure of the cells by a Victoreen Rate Meter with the probe placed in the same position and exposed at the same milliamperage as the cells.

Measurements of clone formation and capacity. Measurements of the cloning efficiency of either UV- or X-irradiated cells were made by trypsinizing the cells immediately after irradiation, washing, and resuspending in fresh medium containing 10% foetal bovine serum. Appropriate dilutions of the cell suspensions were plated on plastic petri dishes in liquid medium. After allowing the cells to settle and attach to the plate, the liquid was removed and replaced by the same nutrient medium containing 0.5% agar. Clones were counted macroscopically 10-15 days later.

Two methods were used to measure the capacity of irradiated cells to support the growth of virus. In the first or direct method,

monolayers of ME cells were irradiated as described and infected immediately. At the higher doses, there was considerable rounding up and loss of cells from the monolayer; therefore, a compensating amount of unirradiated cells was added four hours after the end of virus adsorption. The plates were then overlaid with agar. In the second method, the capacities were measured by replating of infective centers on unirradiated monolayers. The infected, irradiated cells were trypsinized four hours after the end of virus adsorption, washed and replated. In both methods the ability of irradiated, infected cells to give rise to at least one infective virus particle is measured. In the direct method, however, a potential artifact could arise from infection of the newly added cells by residual virus which had not adsorbed to the irradiated cells and was not washed out. Thus the capacity would appear too resistant. In the transfer of infective center method, one might expect too sensitive a capacity to result from a selective injury or loss of irradiated, infected cells due to the trypsinization and centrifugation. In repeated experiments, however, the results obtained by the two methods were in good agreement. The second method was preferred because the same multiplicity of infection could be used for all the points. The results reported here were obtained by the latter method.

RESULTS AND DISCUSSION

1. Inactivations of Py and ME cell capacity by X-rays and UV.

The X-ray inactivation curve of Py is shown in Figure 1. The shoulder was not always observed, and when present varied in number of

hits from 1.1 to 2.5. Multiple infection of single cells cannot account for the shoulder since under conditions of the plaque assay essentially none of the cells adsorbs more than one virus particle. The lethal dose as measured from the straight portion of the curve is 205,000 roentgens. No appreciable effect on the hemagglutinating ability of the virus (6) was observed from a dose of X-rays which inactivated 99.9% of the plaque-forming ability. Since the hemagglutinating property is a function of structures on the viral protein coat, it seems likely that inactivation is due to ionizations in the Py DNA.*

In Figure 2 are shown the X-ray inactivation curves for clone-forming ability and capacity for Py multiplication of ME cells. The capacity curve is complex and multihit, with the most sensitive portion being 60-70 times more resistant than the clone formation.

The UV inactivation curve for Py is given in Figure 3. The curve consists of two components--a sensitive one evident at low doses, and a more resistant one at higher doses. The decrease in sensitivity is roughly 1.8 relative to the initial sensitivity, and after the break there is no further change in rate of inactivation down to a survival of 10^{-5}. Both the position of the break and the magnitude of the change in slope varied somewhat from experiment to experiment. Also plotted in

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*The lethal dose of 205,000 roentgens corresponds to an average of one ionization per 2.7×10^{-18} cm^3 in material of biological composition. This volume is approximately 7% of the volume of the virus particle calculated assuming a sphere of 43 mμ diameter, as suggested from electron micrographs of PTA-stained particles (22). The heavily stained inner core of empty virions has a diameter of 27.5 mμ, corresponding to an inner volume roughly 25% of that of the whole virus. The "ionization" or "inactivation" volume thus appears to fit well into the space occupied by the viral DNA.

Figure 3 is the UV inactivation of Py DNA, which follows closely that of the whole virus for both first and second components. The UV sensitivity of the virus, including the apparent decrease in sensitivity at higher doses, is therefore a property of the DNA itself.

The UV inactivations of cell capacity and clone formation are presented in Figure 4. Again, the capacity curve is multihit, with a steep portion descending over two log units which is close to the curve tracing the loss of clone-forming ability. The zero dose extrapolations of the steep portions of each of the capacity curves (Figs. 2 and 4) falls within the range of 7-10.

2. Analysis of the UV survival curve.

The appearance of the second component is peculiar to the inactivation by UV since single exponential curves are observed for X-ray and also nitrous acid inactivation (2). Four possible factors could give rise to the resistant component:

1) Genetic inhomogeneity in the virus stocks with respect to UV sensitivity. This possibility was ruled out by preparing stocks from four clones (single plaques) of virus which had survived 8 minutes of UV irradiation and repeating the inactivation curve on each. In all four cases, curves identical to the original one, within the normal experimental variation, were obtained.

2) Multiplicity reactivation in cells infected by two or more UV-damaged virus particles. This possibility is ruled out because the dose-response curves for virus receiving various doses are linear.

3) Aggregation of virus particles could give rise to changes in slope. This appears unlikely as a factor here since the curve shows a

single, discrete change in slope. In addition, sonication of the virus suspensions, either before or after irradiation, did not change the shape of the curve.

4) Some type of host cell repair mechanism (HCR) in ME cells which reverses some of the UV lesions in the Py DNA. Results of experiments on phages P22 (7) and λ (8) have been discussed in terms of a repair by recombination between the phage genome and a specific homologous region of the bacterial genome. Subsequent experiments with P22 and T1 (14,15, 16) brought evidence in favor of an enzymatic repair mechanism. This view has been strengthened by the demonstration of an enzyme(s) in bacteria capable of excising thymine dimers from bacterial DNA after UV irradiation (3,17). Repair of UV lesions of irradiated phage also occurs in these bacteria.

Since the ability of bacteria to perform HCR is influenced by their physiological state (15,16), experiments were carried out to see whether the physiological state of ME cells affects the survival of UV irradiated Py.

As a first attempt, cells were irradiated with UV and infected with either irradiated or unirradiated virus. The results are plotted in Figure 5a and b as virus survival curves and capacity curves respectively. Regarded either way, it is evident that survivals of the virus and of the cell capacity are independent of one another. The same is true for X-irradiated virus plated on X-irradiated cells (Figure 5c and d). These experiments therefore fail to provide any evidence for a UV or X-ray sensitive HCR mechanism analogous to that described for P22 (16).

In addition to irradiation, the following treatments had no significant effect on the shape of the survival curve: a) starvation of the cells in Tris-salt buffer with 2% calf serum for 6 hours before infection; b) pretreatment of the cells for 20 hours with 0.2 γ/ml fluoro-deoxyuridine in order to block DNA synthesis temporarily. After adsorption of the virus, the monolayers were overlaid with the normal nutrient agar medium supplemented with thymidine and deoxycytidine. c) preparing the ME cultures at 30°C and allowing the plaques to develop at this temperature. Virus was adsorbed at 37°C as usual.

3. Effect of variation of the eclipse period on the UV survival curve--possible role of HCR.

From the above experiments it appears that the change in slope must be related to some type of physiological heterogeneity in the population of infected cells. A partial elucidation of the nature of this heterogeneity comes from the following experiments, in which the length of the eclipse period is shown to be correlated with the probability of survival.

It has been noted that phage surviving UV irradiation show an extended eclipse period (7,18). To test the possibility of a growth delaying effect of UV on Py, growth curves were determined for irradiated and unirradiated virus (Figure 6). Two doses of UV were chosen, on the basis of many experiments, to lie one on the more sensitive component (2 minutes) and the other on the more resistant component (8 minutes). The multiplicities of infection of survivors were roughly the same for irradiated and unirradiated virus. The monolayers were washed thoroughly

after adsorption to allow a maximum synchrony of infection. The eclipse periods were measured as the time between the end of virus adsorption and the intersection of the total virus curve with the solid line indicating the number of virus-yielding cells. It can be seen that the eclipse periods of lightly irradiated and unirradiated virus are similar while that of heavily irradiated virus is 8-10 hours longer. The increased resistance at higher doses could therefore derive from some kind of HCR mechanism in complexes where the virus growth cycle is delayed.

To test this hypothesis, growth curves and UV inactivation curves were determined under conditions in which the eclipse period was artificially lengthened by inhibiting protein synthesis in infected cells with Puromycin. The inhibitor was added to the culture fluid at a final concentration of 20 γ/ml for a period of 8 hours. The monolayers were then washed, trypsinized, and the cells replated as infective centers. Two widely spaced periods during virus development were chosen for exposure to Puromycin. The results of such a series of experiments are presented in Figures 7, 8 and 9. Complexes exposed early during the eclipse period, from the 3rd to 11th hour, show a delay compared to unexposed complexes, which is roughly equal to the duration of the exposure in the cases of unirradiated and lightly irradiated virus, but little or no delay in the case of heavily irradiated virus (Fig. 7). The survival curve under these conditions is a single exponential with slope corresponding to the less sensitive component (Fig. 9). It appears, therefore, that repair can occur to the same extent in low-dose complexes as in high-dose complexes provided an early inhibition of virus development is brought about.

When the same exposure to Puromycin was given near the end of the eclipse period, from the 20th to the 28th hour, a delay of roughly 25 hours resulted; and the eclipse periods of unirradiated, lightly and heavily irradiated virus were nearly the same (Fig. 8). (The initial rise in these curves is probably related to some asynchrony of infection, the complexes in which development started earliest being ready to release virus already at the time of exposure to Puromycin; this should not affect the conclusion concerning the long delay in the majority of complexes.) No significant change in the UV survival curve is detected under these conditions (Fig. 9). Thus it appears that the factor(s) affecting survival can operate only during the earlier phases of virus development.

Finally, when cells were irradiated with UV prior to infection, no significant changes in the eclipse periods were observed; and, as mentioned above, there was no change in the survival curve (Figs. 5 and 9).

These results can be understood in terms of an enzymatic HCR mechanism in ME cells capable of repairing a certain fraction of the UV-induced lesions in the Py DNA. The fact that repair in low-dose complexes occurs when Puromycin is present during the early phase of virus development (Figure 9) suggests that the enzyme(s) involved is already present in the uninfected cell. When virus is exposed to higher doses, the sublethal damage results in a prolonged eclipse period allowing repair to be more effective. The fact that in the case of the late exposure to Puromycin there is no correlation between extended eclipse period and decreased UV sensitivity indicates that the repair must be performed on the parental (infecting) DNA and therefore must

occur early in the growth cycle before viral DNA synthesis begins (i.e., before approximately 16 hours).

It should be pointed out that the eclipse periods measured in liquid medium may not be the same as under conditions of the plaque assay, where the infected cells are overlaid with nutrient agar. In fact, survival curves based on plaque counts from the 10th-12th day tended to show an increase in resistance, particularly at the lower doses, based on recounting of the plaques on the 18th-20th day; thus, under plaque assay conditions, there may be delays in the development of some of the complexes, thus allowing repair to occur.

4. Test for effects of Puromycin and fixation of UV-damage on capacity.

One might expect variations in the eclipse period to affect the sensitivity of the capacity by allowing more or less time for fixation of radiation damage in those cell structures necessary for virus multiplication. For example, in cases where integrity of the host cell genome is required, processes analogous to those described for "mutation fixation" in bacteria (4) might play a role. This possibility was tested in the two following experiments, in which the capacity of ME cells for unirradiated Py was measured: 1) a nine-hour delay was allowed between the time of UV-irradiation of the cells and infection, and 2) Puromycin (20 γ/ml) was added to the infected, irradiated cells for the first eight hours after infection. An increased sensitivity of the capacity due to each of the treatments could be expected; however, in repeated experiments, this was not observed. Evidence that capacity is concerned with "cytoplasmic" rather than "genomic" integrity has been obtained with

phage λ (Dr. J. Weigle, personal communication). The capacities were measured for multiplication of the phage in two strains of E. coli: HCR^+, which can repair UV lesions in its DNA, and HCR^-, which cannot. The capacities of the two strains were the same, indicating that the critical damage is not in the host DNA.

5. Comparison of the radiobiological behavior of Py and HSV.

The X-ray and UV sensitivities of HSV can be seen in comparison with Py in Figures 1 and 3, respectively. In the case of X-rays, HSV is inactivated exponentially at a rate 6-7 times greater than Py. In the UV inactivation curve of HSV, there is also a change in slope, which is by a factor of roughly 5.5 compared to 1.8 for Py. With UV, the ratio of the initial slopes (HSV to Py) is approximately 17, in good agreement with the ratio of the DNA contents--68×10^6 for HSV (13), and 3×10^6 for Py (20). Thus, the intrinsic UV sensitivity of Py is not unusually low when compared to HSV. With X-rays, the intrinsic sensitivity of Py is higher than that of HSV. It should be noted, however, that a greater X-ray sensitivity of HSV than that observed here has been reported (9); the reasons for the discrepancy are not known.

The X-ray capacity curve for HSV is very similar to that for Py as can be seen in Figure 2. The UV capacity curve for HSV (Fig. 4) is also similar in shape to that for Py, but more resistant at all doses tested. The ratio of surviving infecting centers HSV to Py appears to increase at low doses and then remain constant. In general, the capacities of ME cells for the two viruses are not widely different. Thus, neither the results on intrinsic sensitivities of Py and HSV, nor the relationship between the cell capacities for these two viruses bears out the

expectations from similar studies with temperate and virulent bacteriophage. In addition, the data presented in Figure 5a-d demonstrate a further breakdown in the Py-temperate phage analogy since the capacity of bacteria has been shown to be more sensitive for irradiated than unirradiated phage in the case of several temperate phage (18).

CONCLUSIONS

1. The survival curve for UV-irradiated Polyoma virus (Py) on mouse embryo (ME) cells normally consists of two exponential components. The survivors of higher doses show an increase in eclipse period of approximately 30% compared to unirradiated or lightly irradiated virus. If a comparable delay is brought about in all of the complexes by adding Puromycin during the first part of the eclipse period, the survival curve becomes a single exponential with the sensitivity of the less sensitive component. These results suggest an enzymatic repair mechanism in ME cells which operates more effectively if virus development is delayed.

2. The effect of Puromycin in bringing about a delay in the eclipse period depends on the stage during virus development at which the inhibitor is added. Thus, exposure between the 3rd and 11th hour, during the expression of "early" viral functions, causes a 7-hour delay, while exposure between the 20th and 28th hour, during the expression of "late" virus functions, causes roughly a 25-hour delay. The latter result suggests that the structures responsible for early functions decay at an appreciable rate and must be resynthesized if late functions are blocked. This interpretation has been applied to the results of similar experiments with HSV (10). Attempts are currently being made to test

this hypothesis by studying the behavior of Py-specific messenger RNA in infected mouse cells.

3. The experiments on intrinsic virus sensitivities and host cell capacities for the multiplication of Py and HSV have shown more similarities than differences between these two different viruses. In particular, Py, which is well known for its oncogenic properties, exhibits little of the behavior expected of a "temperate" virus. It seems, therefore, that similar studies of the cytolytic interaction of other viruses suspected of oncogenic potential would probably be of no predictive value.

Acknowledgement

The author wishes to express his gratitude to Dr. Renato Dulbecco for many useful discussions during the course of this work and for his help in the preparation of the manuscript. He wishes also to acknowledge the very helpful technical assistance of Mrs. Laverne Wenzel, Mrs. Arger Drew and Mrs. Benneta Keeley.

REFERENCES

1. Ackerman, P. H., and Black, F. L. (1961). A radiological analogy between measles virus and temperate phages. Proc. Natl. Acad. Sci. U.S. 47, 213-220.

2. Benjamin, T. L. (1965). Relative target sizes for the inactivation of the transforming and reproductive abilities of Polyoma virus. Proc. Natl. Acad. Sci. U.S. 54, 121.

3. Boyce, R. P. and Howard-Flanders, P. (1964). Release of ultraviolet light-induced thymine dimers from DNA in E. coli K-12. Proc. Natl. Acad. Sci. U.S. 51, 293-300.

4. Doudney, C. O., and Haas, F. L. (1959). Gene replication and mutation induction in bacteria. J. Mol. Biol. 1, 81-83.

5. Dulbecco, R., and Freeman, G. (1959). Plaque production by polyoma virus. Virology 8, 396-397.

6. Eddy, B. E., Rowe, W. B., Hartley, J. W., Stewart, S. E., and Huebner, R. J. (1958). Hemagglutination with the SE Polyoma virus. Virology 6, 290-291.

7. Garen, A., and Zinder, N. (1955). Radiological evidence for partial genetic homology between bacteriophage and host bacteria. Virology 1, 347-376.

8. Kellenberger, G., and Weigle, J. (1958). Etude au moyen des rayons ultraviolets de l'interaction entre bactériophage et bactérie hôte. Biochim. Biophys. Acta 30, 112-124.

9. Powell, W. F. (1959). Radiosensitivity as an index of herpes simplex virus development. Virology 9, 1-19.

10. Roizman, B. (1963). The programming of herpes virus multiplication in double-infected and in puromycin-treated cells. Proc. Natl. Acad. Sci. U.S. 49, 165-171.

11. Rubin, H., and Temin, H. M. (1959). A radiological study of cell-virus interaction in the Rous Sarcoma. Virology 7, 75-91.

12. Rubin, H. (1960). Growth of Rous Sarcoma Virus in chick embryo cells following irradiation of host cells or free virus. Virology 11, 28-47.

13. Russell, W. C., and Crawford, L. V. (1964). Properties of the nucleic acids from some herpes group viruses. Virology 22, 288-292.

14. Sauerbier, W. (1962a). Evidence for a non-recombinational mechanism of host cell reactivation of phage. Virology 16, 398-404.

15. Sauerbier, W. (1962b). The influence of the metabolic state of the host bacteria on the survival of phage T1 irradiated with ultraviolet light. Virology 17, 164-170.

16. Sauerbier, W. (1962c). The bacterial mechanism reactivating ultraviolet-irradiated phage in the dark. Z. Vererbungslehre 93, 220-228.

17. Setlow, R. B., and Carrier, W. L. (1964). The disappearance of thymine dimer from DNA: an error-correcting mechanism. Proc. Natl. Acad. Sci. U.S. 51, 226-231.

18. Stent, G. S. (1958). Mating in the reproduction of bacterial viruses. Advances in Virus Research 5, 95-150.

19. Stoker, M., and Abel, P. (1962). Conditions affecting transformation by polyoma virus. Cold Spring Harbor Symposium on Quantitative Biology 27, 375-386.

20. Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P. (1965). The twisted circular form of polyoma viral DNA. Proc. Natl. Acad. Sci. U.S. 53, 1104-1111.

21. Vogt, M., and Dulbecco, R. (1960). Virus-cell interaction with a tumor-producing virus. Proc. Natl. Acad. Sci. U.S. 46, 365-370.

22. Watson, J. D. (1950). The properties of x-ray-inactivated bacteriophage. J. Bacteriology 60, 697-718.

23. Weil, R. (1961). A quantitative assay for a sub-viral infective agent related to polyoma virus. Virology 14, 46-53.

24. Wildy, P., Stoker, M., Macpherson, I., and Horne, R. W. (1960). The fine structure of polyoma virus. Virology 11, 454-457.

25. Winocour, E. (1963). Purification of polyoma virus. Virology 19, 158-168.

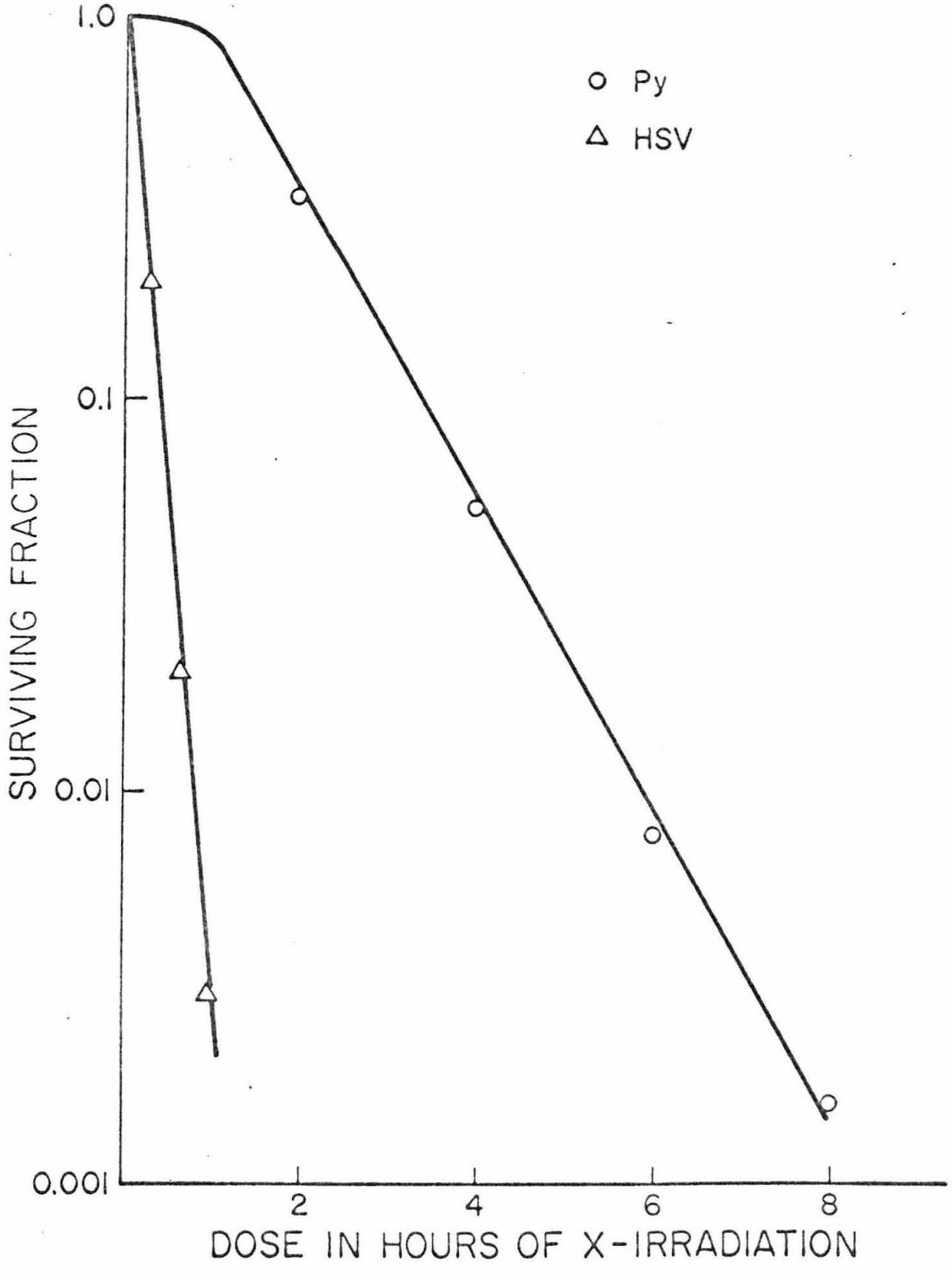

Figure 1. X-ray inactivation of Py and HSV.

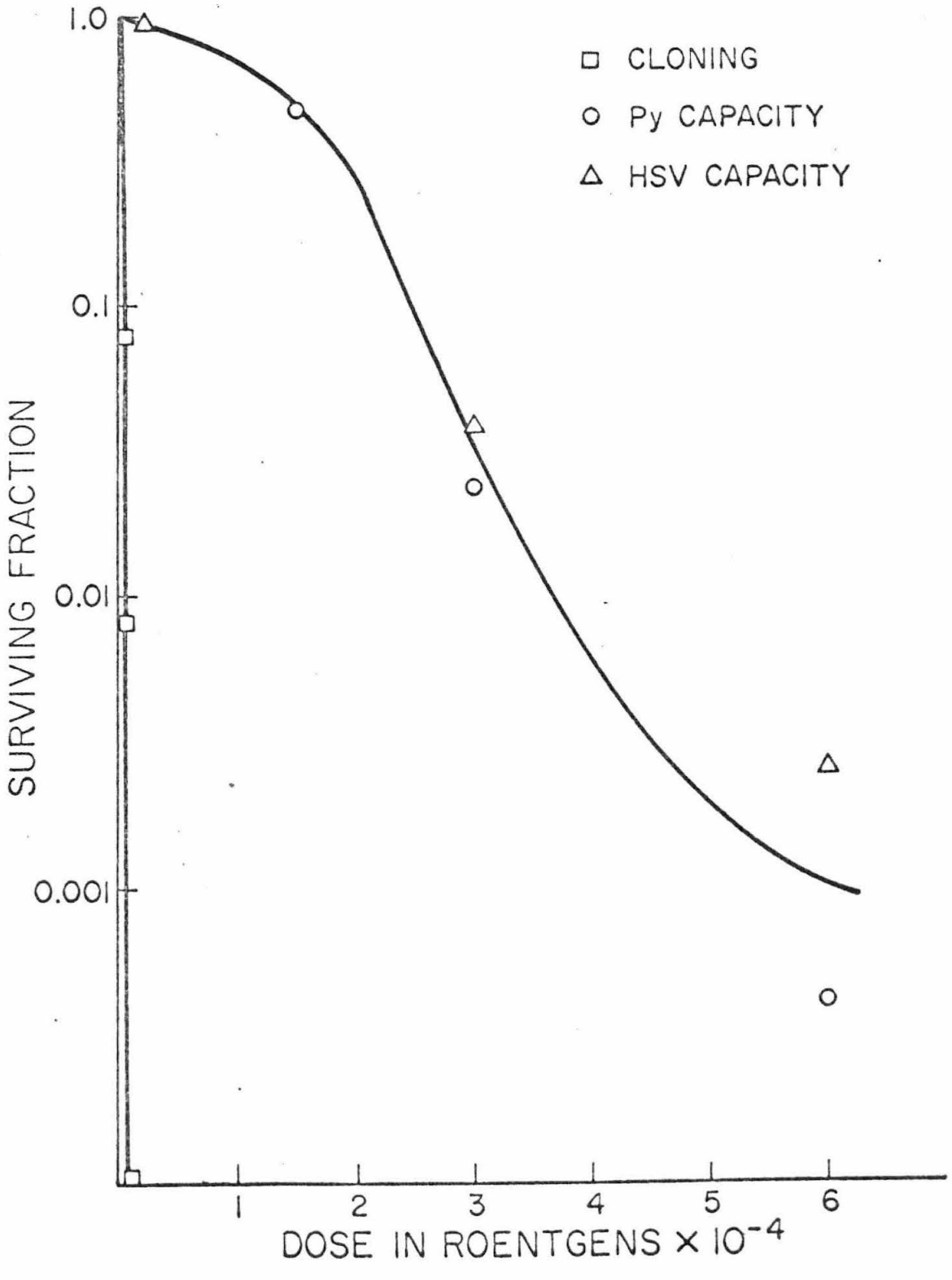

Figure 2. X-ray capacity and cloning curves.

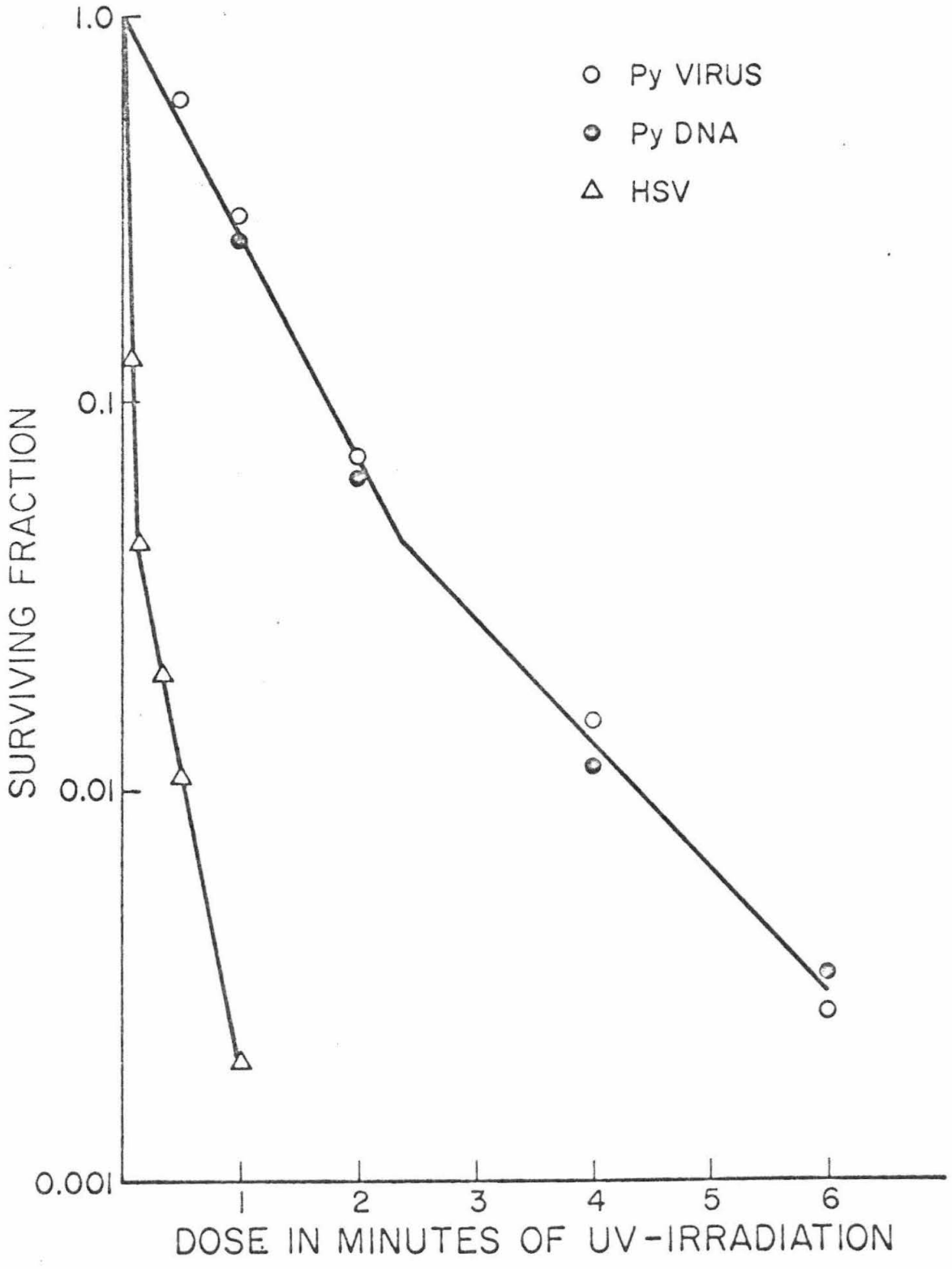

Figure 3. UV inactivation of Py and HSV.

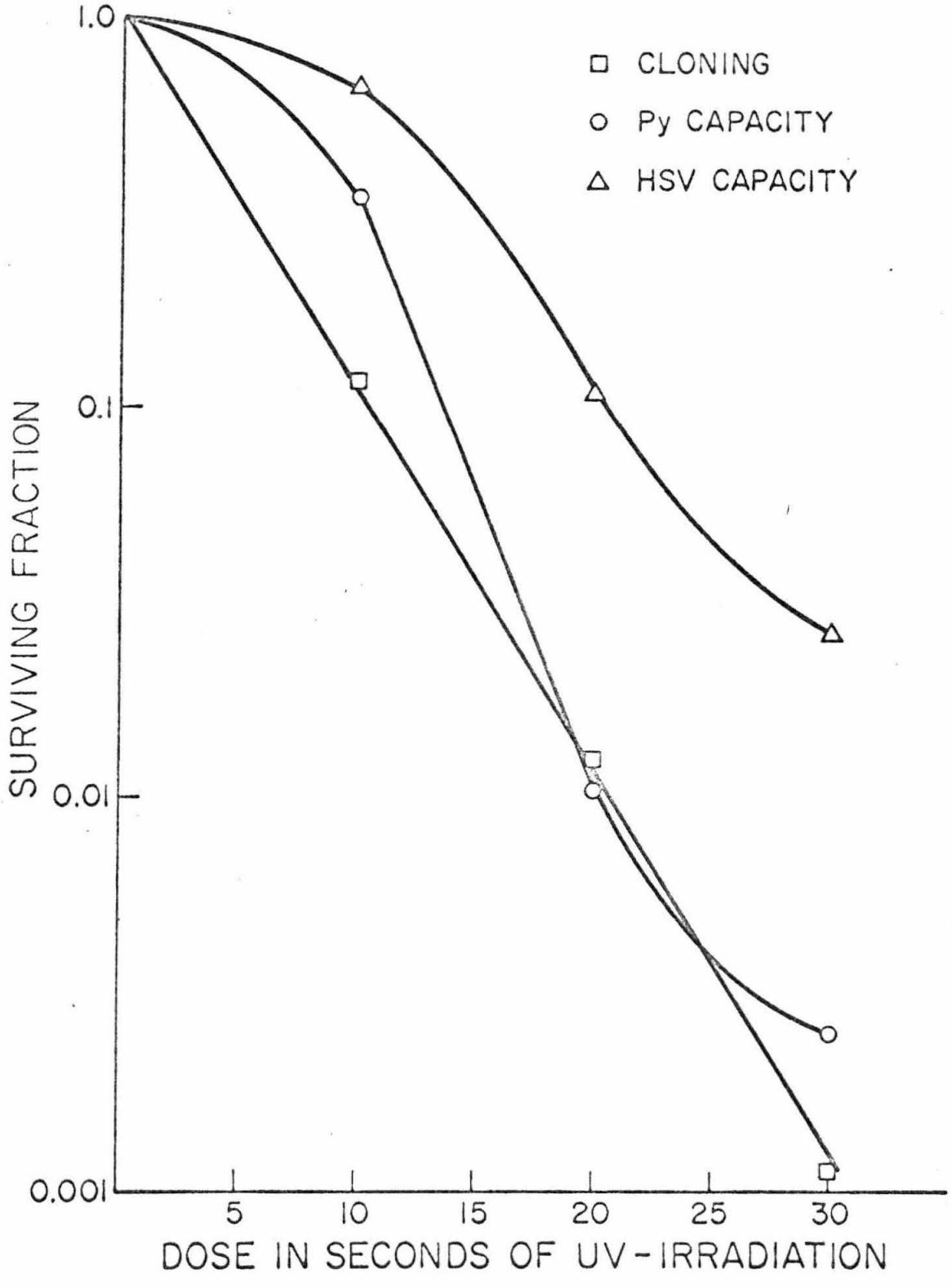

Figure 4. UV capacity and cloning curves.

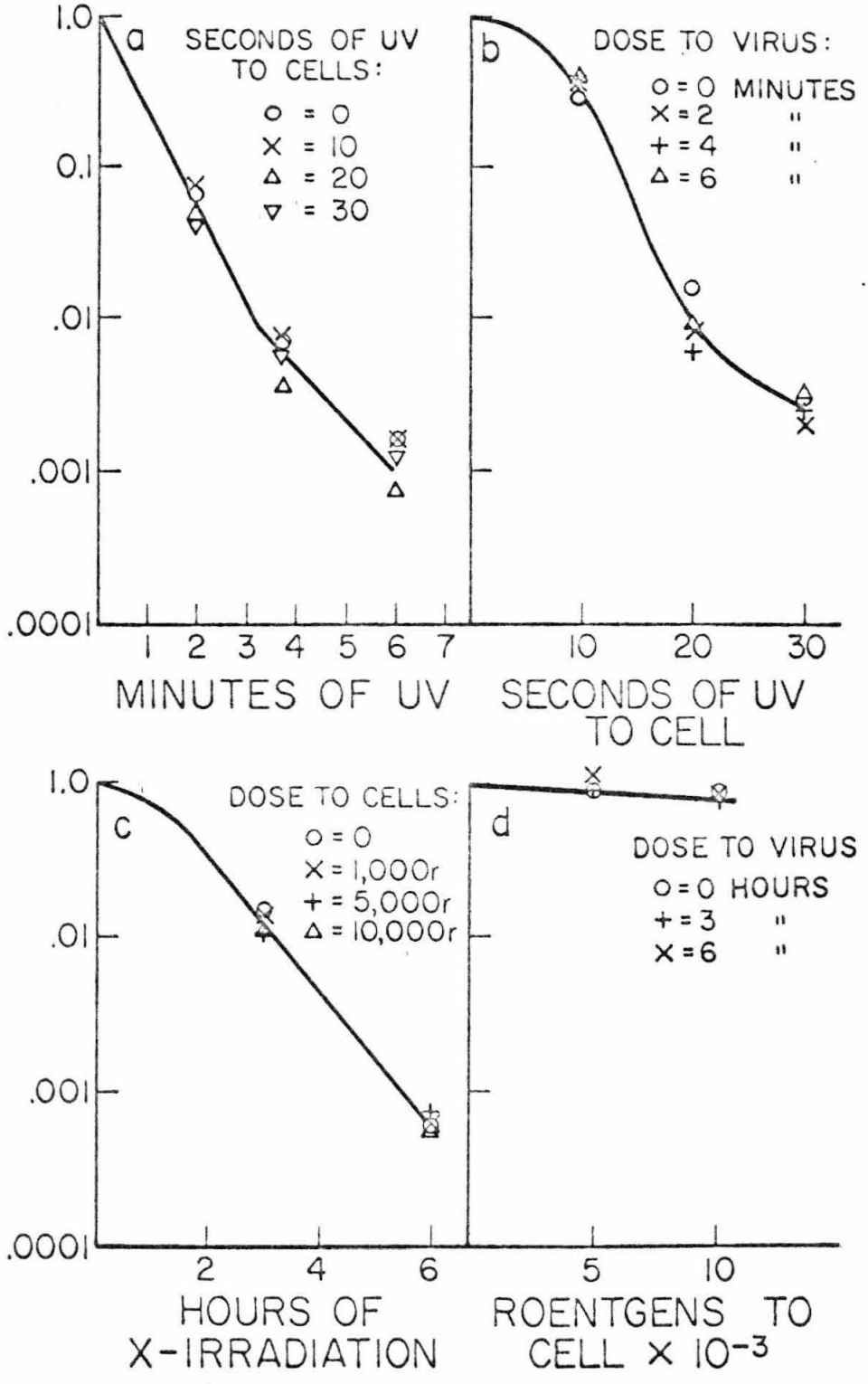

Figure 5. a) UV survival of Py on UV-irradiated cells. b) UV capacity for UV-irradiated Py. c) X-ray survival of Py on X-irradiated cells. d) X-ray capacity for X-irradiated Py.

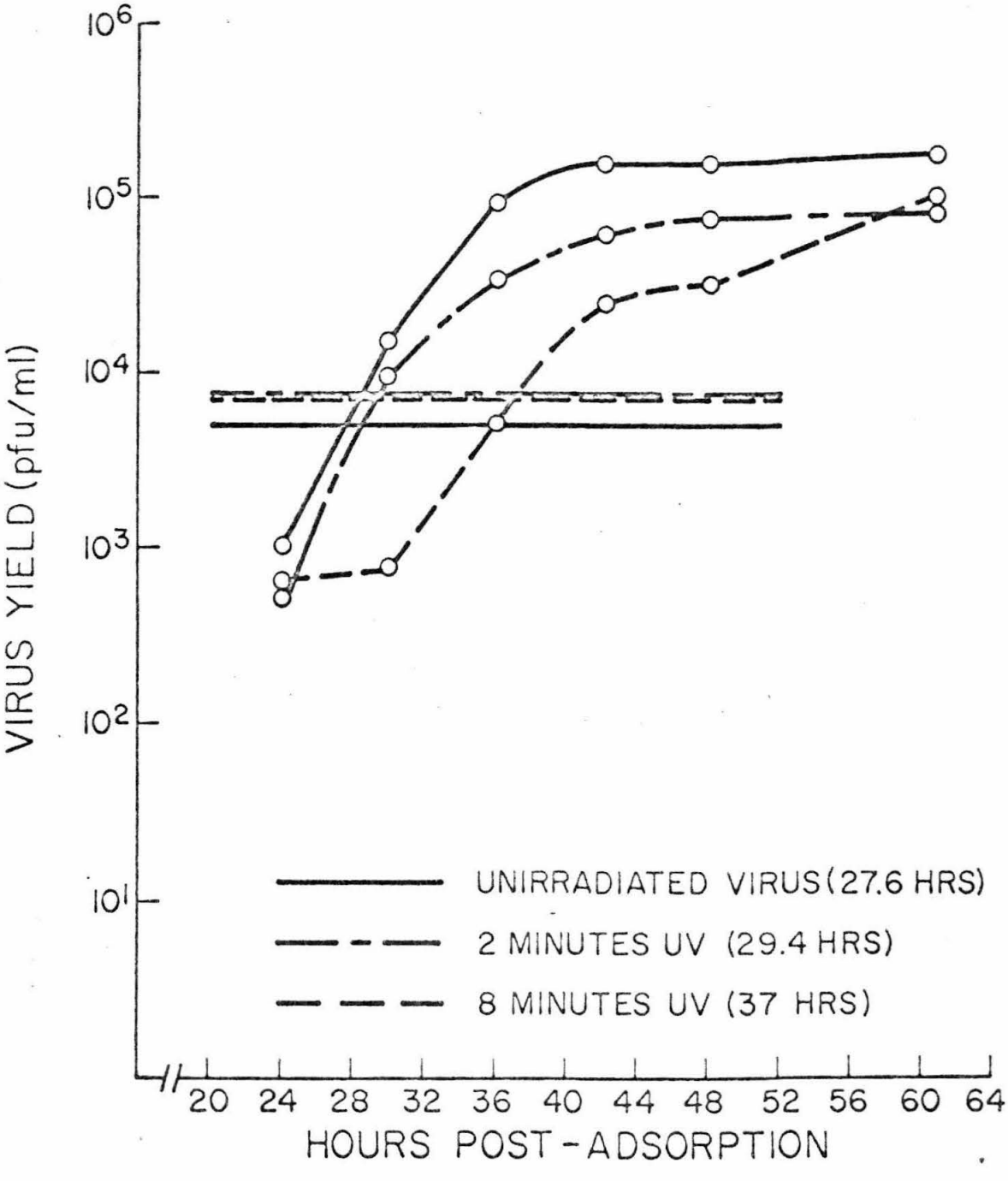

Figure 6. Growth curves of UV-irradiated and unirradiated Py. The fractional survivals were 1.0, 0.15, and 0.0014 for 0, 2, and 8 minutes of irradiation, respectively. Values in parentheses indicate the length of the eclipse period for each case.

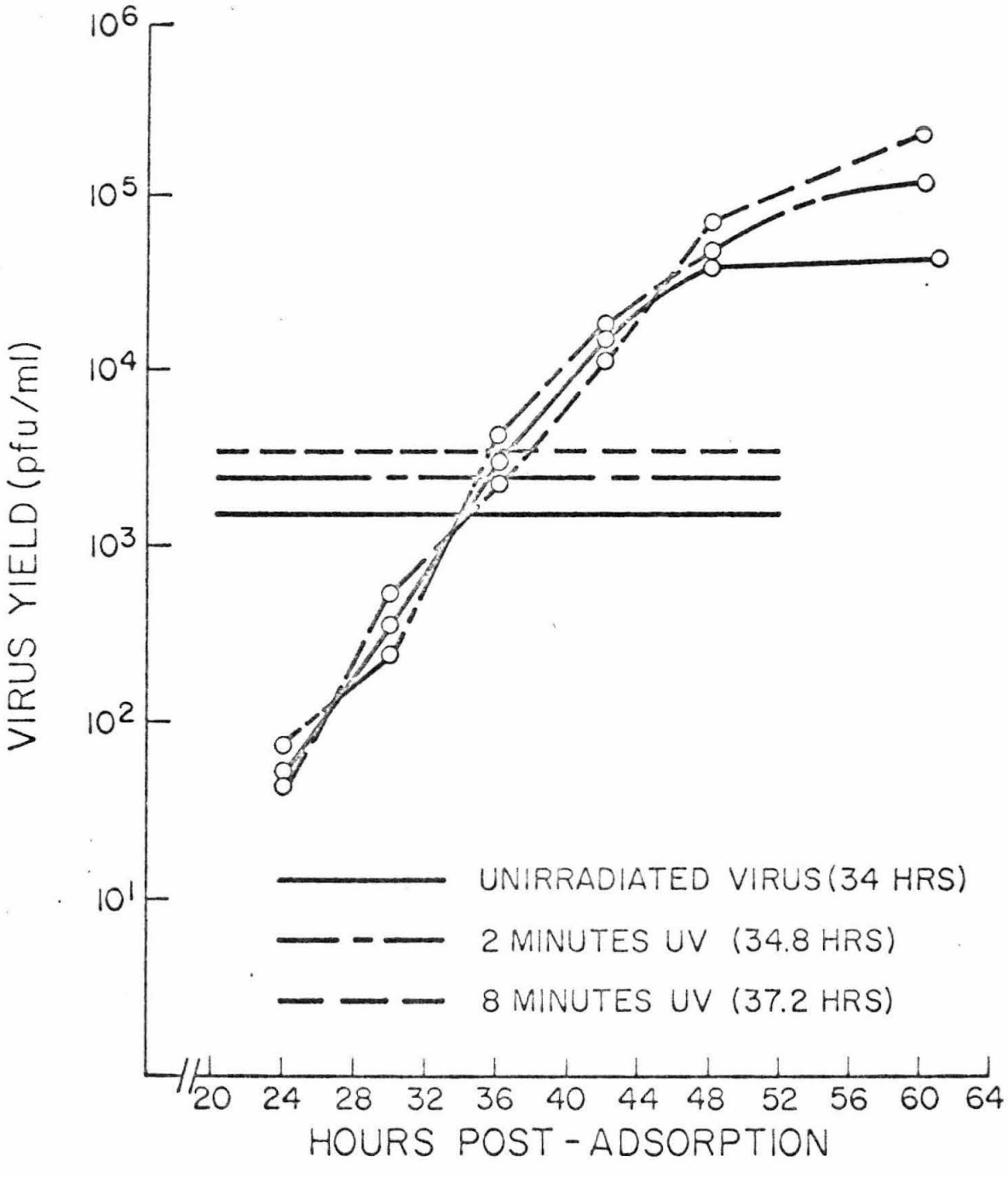

Figure 7. Growth curves of UV-irradiated and unirradiated Py with Puromycin between 3rd and 11th hours. The fractional survivals were 1.0, 0.15, and 0.0014 for 0, 2, and 8 minutes of irradiation, respectively. Values in parentheses indicate the length of the eclipse period for each case.

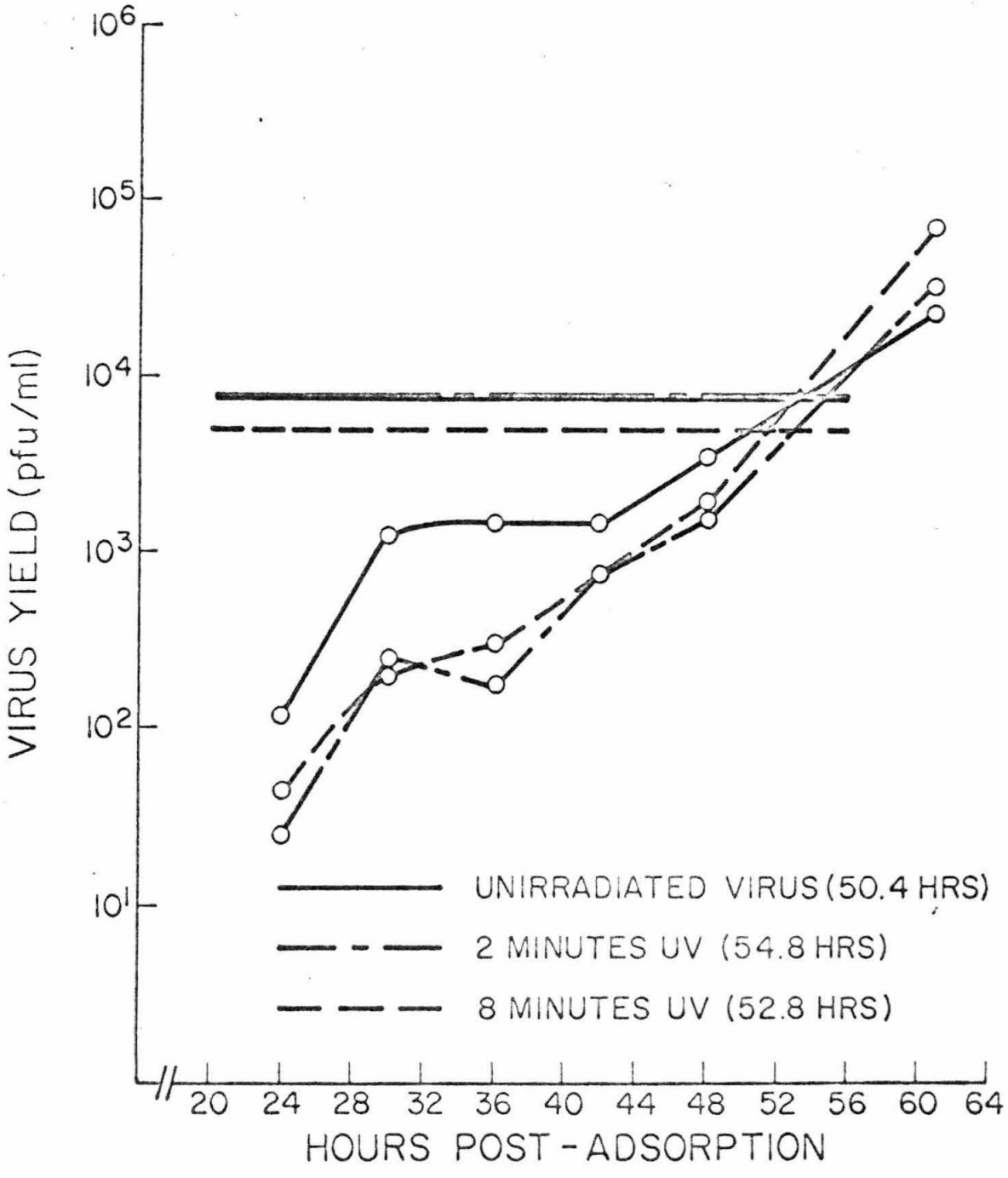

Figure 8. Growth curves of UV-irradiated and unirradiated Py with Puromycin between 20th and 28th hours. The fractional survivals were 1.0, 0.15, and 0.0014 for 0, 2, and 8 minutes of irradiation, respectively. Values in parentheses indicate the length of the eclipse period for each case.

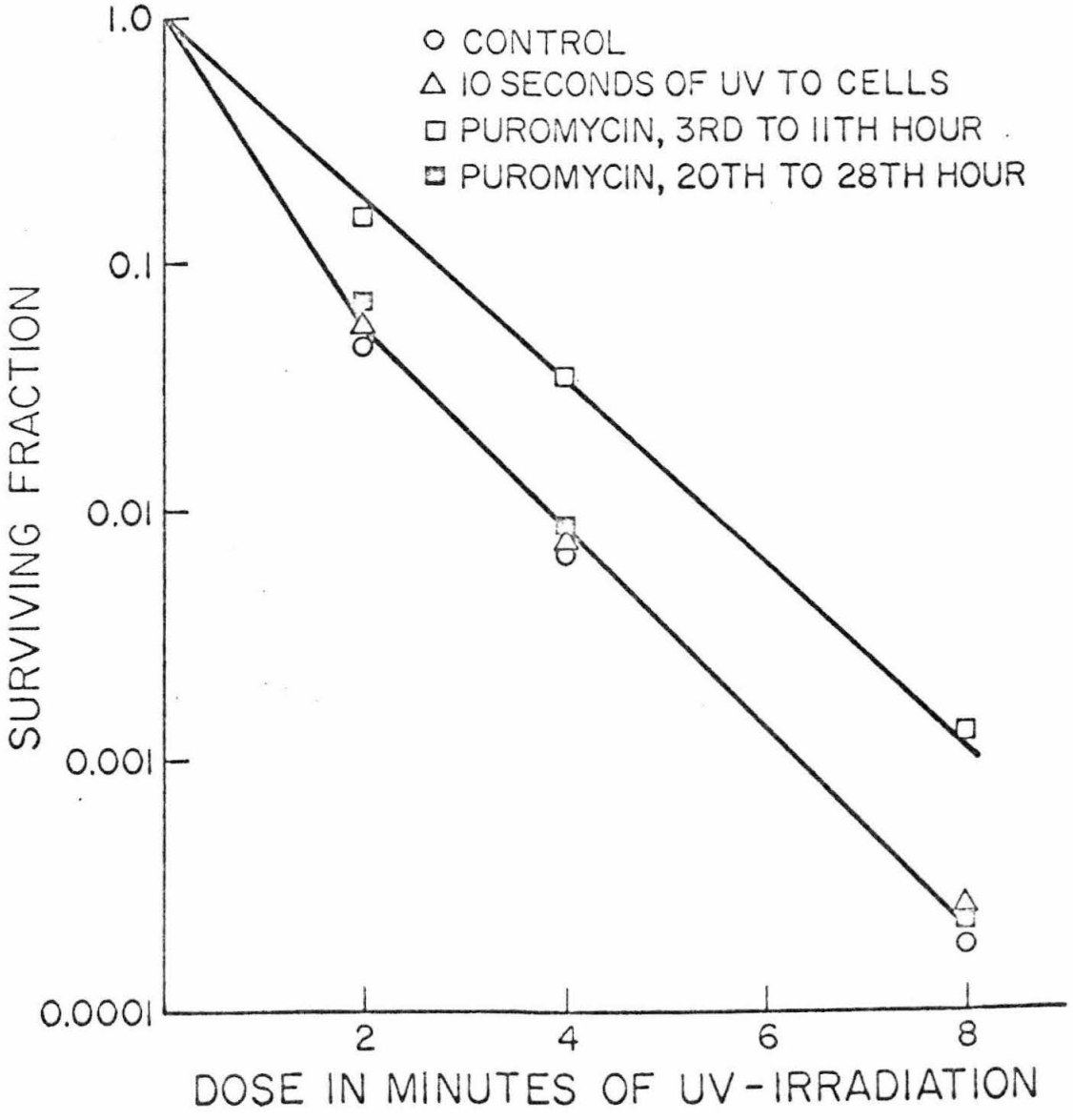

Figure 9. UV survival curve of Py on cells exposed to UV or Puromycin.

P A R T II

RELATIVE TARGET SIZES FOR THE INACTIVATION OF THE TRANSFORMING AND REPRODUCTIVE ABILITIES OF POLYOMA VIRUS*

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Infection by polyoma virus of tissue culture cells causes in mouse embryo cells a cytocidal interaction with virus multiplication (1,2), and in hamster cells a non-cytocidal interaction resulting in the acquisition by the cell of permanently altered growth characteristics akin to those of malignant cells (3,4). The latter process, referred to as transformation, is generally regarded as the in vitro counterpart of the process whereby polyoma causes tumors in animals. It is not known whether all of the genes present in the polyoma virus DNA are required for transformation. This problem has now been studied by comparing the inactivation rates, or target sizes, of polyoma virus in regard to its cytocidal (plaque forming) and transforming effects. Four different methods of inactivation have been used -- ultraviolet and X-irradiation, nitrous acid, and P^{32}-decay. With each of these agents, the target size of the transforming ability was found to be between 55 and 65% that of the cytocidal ability.

Materials and Methods. The small plaque variety of polyoma virus was grown and purified according to Winocour (5).

Assays: The standard plaque assay on mouse embryo cells was used (6). Transformation was measured on BHK (baby hamster kidney) cells by using the agar suspension method (7).

Inactivations: Procedures for UV and X-irradiations are described elsewhere (8). Total emission from a germicidal lamp was used as the UV source. The dose rate corresponds approximately to 50 lethal hits per minute to bacteriophage T2 in the absence of photoreactivation. 220-KV X-rays filtered through 1 mm of aluminum were used at a dose rate of approximately 3250 roentgens per minute. Nitrous acid inactivation was carried out by mixing equal volumes of 2 M $NaNO_2$, 1 M acetate buffer pH 4.5, and virus suspension. The reaction was allowed to proceed at room temperature for the times indicated, then stopped by rapid cooling and addition of an equal volume of cold 5 M Tris buffer pH 8.5, followed by dialysis against standard saline citrate buffer. P^{32}-labeled virus was prepared as follows: Confluent baby mouse kidney cultures were kept in phosphate-free medium for 24 hr before infection. The cultures were infected at a multiplicity of 10-50 pfu/cell, washed free of unadsorbed virus, and then incubated in Eagle's medium containing 10^{-5} M phosphate and 5% dialysed horse serum. RDE (receptor destroying enzyme) and anti-polyoma antiserum were added to the cultures from the 6th to 10th hours after infection to remove residual cell-associated virus. The cultures were again washed and re-incubated in the same low phosphate medium. At the 14th hour after infection, carrier-free P^{32}-orthophosphate was added in amount sufficient to make the specific activity of the medium approximately 1 P^{32}:200 P^{31}. The cultures were harvested at the 50th hour, the cells sonicated to release the virus, and the debris removed by low speed centrifugation. The free P^{32} was removed by dialysis, and the virus stored at -70°C for

periodic assay. Unlabeled virus prepared under the same conditions, except for the omission of the isotope, was used as a control. To compensate for variations in the sensitivities of the assays from week to week, the survival values were taken as ratios of the titer of labeled to that of unlabeled virus as determined in the same assay.

Results and Discussion: In Figure 1 (a-d) are presented typical survival curves for the plaque forming and transforming abilities of polyoma virus for each kind of inactivation. In each case, the inactivation is a single hit exponential process. The change in slopes of the UV inactivation curves at higher doses is not important for the present discussion; its nature will be discussed elsewhere (8). Only the initial slopes are considered here. For any of the methods of inactivation, the ratio of the slopes of the survival curves is a direct measure of the relative target sizes of the virus for the two effects. In repeated experiments, the values for this ratio (transformation: plaque formation) were always between 0.54 and 0.66 and, within these limits, did not depend on the kind of inactivation.

The consistently observed difference in target size suggests that the amount of viral genome required for transformation is approximately 55 - 65% of the amount required for virus reproduction. The results, however, do not bear on the question of viral genes common to both types of interaction, i.e., the extent to which the two targets overlap. The interpretation in terms of the relative amounts of viral genetic material required for the two effects rests on two basic assumptions: 1) The DNA of the virus is the target of inactivation in each case, and

2) The probabilities of survival of viral function are not differentially modified by interaction with the two host cell species used.

The first assumption seems justified for the following reasons: 1) The extracted viral DNA has both plaque forming (9) and transforming (10,11) abilities; 2) The rates of UV inactivation of the plaque forming ability of the virus and of the viral DNA are indistinguishable (8); 3) The doses of X-rays used here do not affect the hemagglutinating ability of the virus which is a function of the viral protein, and 4) The primary effect of P^{32}-decay is restricted essentially to the DNA which contains most if not all of the isotope.

The major uncertainty in the interpretation stems from the use of different cell species in the two assays. The results could be influenced by two classes of cellular factors: 1) Enzymatic repair of the lesions in the polyoma virus DNA. Such repair is known to be extensive for UV damages (12), much less in the case of X-rays (13), and is not known for either nitrous acid (12) or P^{32}-decay. The ratios of the target sizes as determined by the various agents, however, are the same; therefore, it is unlikely that such repair plays any role; 2) Differences in the "code dictionaries" between mouse and hamster cells. Such differences would be relevant only if the induction of conditional lethal mutations (14) was an important mechanism of inactivation, and if some of these mutations were lethal in mouse but not in hamster cells. This could occur in virus inactivated by nitrous acid, UV, or X-rays, which are known to be mutagenic, although mutagenesis is unlikely to be a significant cause of inactivation in the case of X-rays. Decay of

P^{32} has not been reported to be mutagenic for bacterial viruses. Evidence against a differential effect by the host cells was also obtained in an experiment in which the survival of the reproductive ability of polyoma virus DNA was measured in the small proportion of hamster cells in which a cytocidal interaction occurs. Hamster and mouse embryo secondary cultures were infected with unirradiated or UV-irradiated viral DNA, and the cells replated as infective centers on mouse embryo secondary cultures. The infected cells were treated just prior to transfer with a dose of X-rays sufficient to destroy their ability to divide without affecting their capacity to support virus multiplication. With a single dose of UV to the polyoma virus DNA, the fractional survival was 0.36 ± 0.09 on mouse embryo cells, and on two preparations of hamster embryo cells 0.39 ± 0.07 and 0.33 ± 0.1. Since both enzymatic repair and mutational inactivation are perhaps most likely in UV-treated virus, this result shows that these phenomena do not play a major role in the target size determinations.

Multiplicity reactivation could conceivably contribute to the difference in inactivation rates, particularly in view of the higher virus to cell ration required for transformation. This could not be a major factor, however, because in both assay systems the dose response curves of virus inactivated by the various agents were linear.

Summary. The rate of inactivation of the transforming ability of polyoma virus has been found to be 55-65% of the rate of inactivation of the reproductive ability using four different methods of inactivation. These results have been interpreted to mean that transformation requires

the participation of only 55-65% as much of the viral DNA as does plaque formation.

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References

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1. Eddy, B. E., S.E. Stewart, and W. Berkeley, Proc. Soc. Exptl. Biol. Med., 98, 848 (1958).

2. Sachs, L., M. Fogel, and E. Winocour, Nature, 183, 663 (1959).

3. Vogt, M., and R. Dulbecco, these PROCEEDINGS, 46, 365 (1960).

4. Macpherson, I., and M. Stoker, Virology, 16, 147 (1962).

5. Winocour, E., Virology, 19, 158 (1963).

6. Dulbecco, R., and G. Freeman, Virology, 8, 396 (1959).

7. Macpherson, I., and L. Montagnier, Virology, 23, 291 (1964).

8. Benjamin, T., in preparation.

9. Weil, R., Virology, 14, 46 (1961).

10. Crawford, L., R. Dulbecco, M. Fried, L. Montagnier, and M. Stoker, these PROCEEDINGS, 52, 148 (1964).

11. Bourgaux, P., D. Bourgaux-Ramoisy, and M. Stoker, Virology, 25, 364 (1965).

12. Sauerbier, W., Virology, 16, 398 (1962).

13. Winkler, U., Virology, 24, 518 (1964).

14. Epstein, R.H., A. Bolle, C.M. Steinberg, E. Kellenberger, E. Boy de la Tour, R. Chevalley, R. S. Edgar, M. Susman, G.H. Denhardt, and A. Lielausis, Cold Spring Harbor Symp. Quant. Biol., 28, 375 (1964).

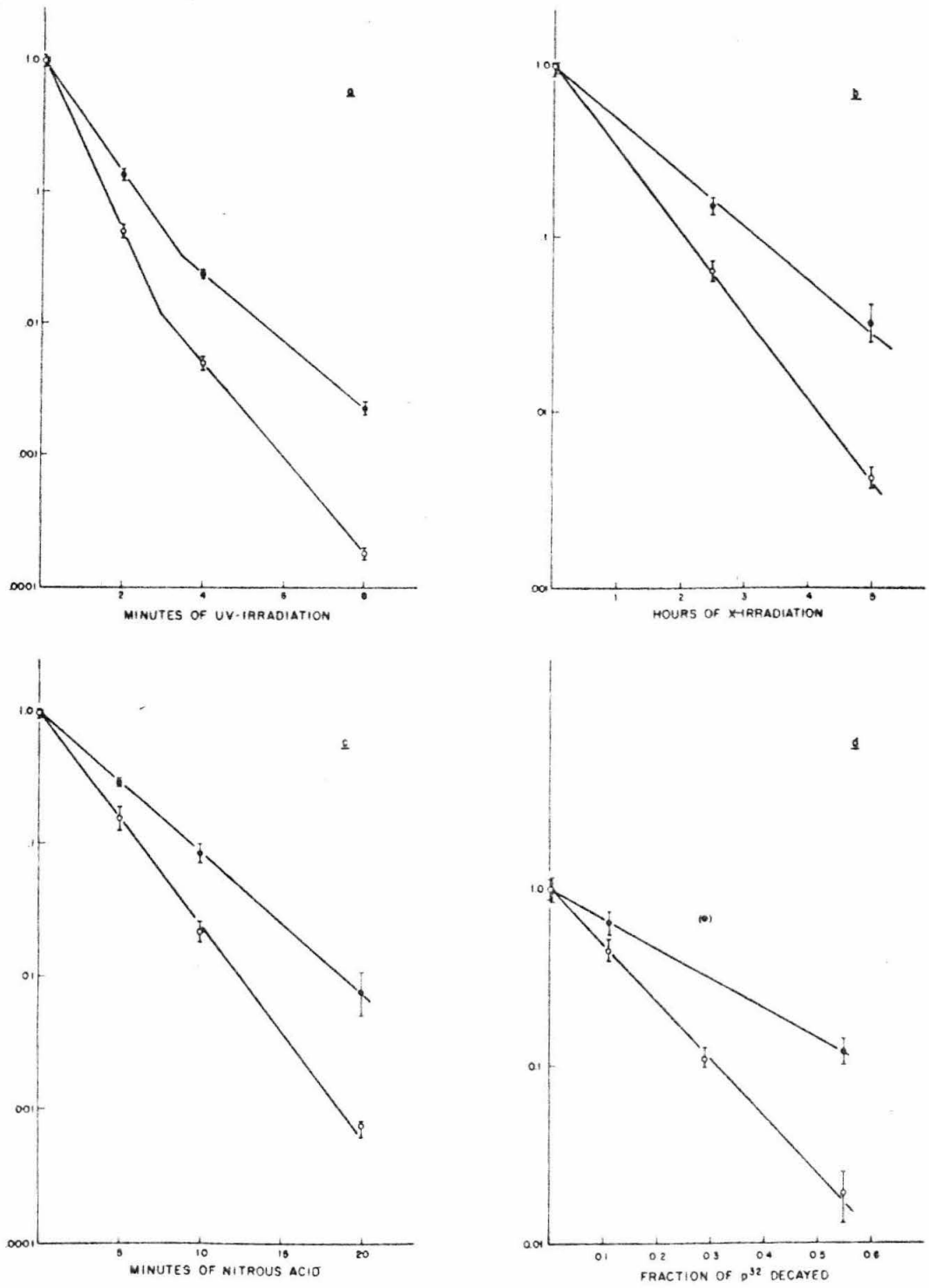

Fig. 1 - Inactivation of plaque-forming (○) and transforming (●) abilities of polyoma virus by a) ultraviolet light, b) X-rays, c) nitrous acid, and d) P-32 decay. Logs of surviving fractions are on the ordinates. Brackets indicate 95% confidence limits.

P A R T III

Virus-specific RNA in Cells Productively Infected or Transformed by Polyoma Virus

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Virus-specific RNA is detected in mouse kidney cells infected by polyoma virus by means of hybridization between H^3-thymidine-labelled virus DNA and P^{32}-pulse labelled RNA. Small amounts of "early" RNA are observed before virus DNA synthesis begins; during the later phases of virus development, there is a sharp increase in the amount of virus-specific RNA. The "late" RNA appears to saturate only a fraction of the sites on the virus DNA. Synthesis of mouse RNA occurs, and is possibly stimulated, during the infection.

A small fraction of the pulse-labelled RNA from virus-free polyoma transformed cells is capable of hybridizing with the virus DNA. No such hybridization is observed with RNA from normal or other malignant cells. This finding provides evidence for the persistence of viral genome in the transformed cell.

1. Introduction

The problem of demonstrating the presence of virus-related materials in mammalian cells transformed (i.e., rendered neoplastic or "malignant") by viruses of the polyoma - SV-40 group has been studied in recent years by means of several experimental approaches (Vogt & Dulbecco, 1962). Conclusive evidence on this point would be critical to the formulation of acceptable theories of viral carcinogenesis by these DNA tumor viruses.

Evidence for virus-specific complement-fixing antigens in tumor or transformed cells has been shown for polyoma (Habel, 1965), for SV-40 (Black, Rowe, Turner & Huebner, 1963), and for adenovirus types 12 and 18 (Huebner, Rowe, Turner & Lane, 1963). In addition, virus-specific transplantation antigens have been shown for polyoma and SV-40 (Sjogren, Hellestrom & Klein, 1961; Habel & Eddy, 1963; Koch & Sabin, 1963; and Defendi, 1963). Other results have indicated that the genetic determinant for the polyoma transplantation antigen can be "rescued" by superinfection of transformed cells with appropriately marked virus strains (Ting, 1964). Although strongly suggestive of the continued presence of viral genes, these results alone are not directly conclusive in this regard.

Using techniques of nucleic acid hybridization, attempts have been made to reveal a complementarity of base sequence between DNA of polyoma transformed cells and DNA of the virus (Axelrod, Bolton & Habel, 1964) or RNA made from the viral DNA by the DNA-dependent RNA polymerase from E. coli (Winocour, 1965). Although a greater affinity of the viral DNA

for DNA of transformed cells as compared to that of normal cells was observed, the results were somewhat obscured by a variable degree of homology with normal cellular DNAs. In the studies with synthetic viral RNA, no differences between normal and transformed cell DNA were observed, which would have been detected if roughly 20 viral DNA equivalents per cell were present.

In the present investigation, hybridization experiments are performed using purified viral DNA and RNA made in vivo by normal and transformed cells. As a preliminary step toward the detection of virus-specific RNA in transformed cells, conditions were first sought by which virus-specific RNA in cells infected by and releasing polyoma virus could be detected and characterized. The results show a progressive increase in the amount of virus-specific RNA during virus development. Using the same procedure, it was possible to detect a small fraction of the RNA in polyoma transformed cells, but not in normal or other malignant cells, capable of hybridizing with polyoma DNA.

2. Materials and Methods

(a) Preparation of H^3-thymidine-labelled viral DNA

Py was obtained from infected mouse kidney cultures as previously described (Winocour, 1963). On the 2nd or 3rd day after preparing the cultures, 10 ml. of fresh medium containing 2 μcuries/ml. of H^3-thymidine (New England Nuclear Corp., specific activity 1.4×10^4 μcuries/μmole) was added to each culture dish (100 x 20 mm). Cultures were harvested on the 5th or 6th day.

Concentration and purification of the virus from the crude lysates were carried out either by centrifugation in the S30 Spinco rotor for 2 hrs at 28,000 rev./min followed by treatment of the pellet with 0.5% sodium deoxycholate (Murakami, 1963), or by precipitation with absolute methanol (33% final volume) in the cold. The methanol precipitate was taken up in a neutral Tris buffer containing 0.025% trypsin, and treated with 0.5% sodium deoxycholate. The partially purified virus was then treated with RNase and DNase (10 γ/ml. each) for 1 hr at 37°C, spun down and resuspended in a CsCl solution of density 1.33 gm/ml. The mixture was spun in the SW39 rotor for 18-24 hrs at 30-35,000 rev./min. The virus band was collected, dialyzed against Tris-buffered saline and stored for extraction at -70°C. The DNA of the virus was extracted with phenol (Weil, 1961).

Analyses of the DNA by band sedimentation through neutral or alkaline CsCl were performed in the Spinco Model E ultracentrifuge to determine the distribution of the components (Vinograd, Lebowitz, Radloff, Watson, and Laipis, 1965). Since even a minor contamination of the polyoma DNA by mouse DNA could obscure the significance of the hybridization results, the DNA preparations were fractionated on sucrose density gradients at neutral pH in order to isolate the major viral components (I and II) from the small amount of heterogeneous component (III). In one preparation used in these studies, only components I and III were initially present (roughly 95% and 5%, respectively), and in the other preparation component II was present (roughly 20%) in addition to I and III. After fractionation, pure component I was obtained from the first preparation, and a mixture of

components I and II from the second; no component III could be detected by analytical ultracentrifugation in either of the final preparations. The specific activity of the viral DNA, counted under the conditions used for hybrid detection, was 4.5 - 7.3 x 10^3 cts/min/γ.

(b) Preparation of P^{32}-labelled RNA

Baby mouse kidney cultures were incubated in phosphate-free Eagle's medium for 24 hours prior to infection. Cultures were infected at a multiplicity of 10-20 pfu/cell, washed, and incubated in a low phosphate medium (2.5 x 10^{-5} M, compared to 2 x 10^{-3} M for normal medium). At different times after infection, 0.2-0.3 millicurie of carrier-free P^{32} orthophosphate was added to each culture containing 10 ml. of medium. Either four or eight hours after addition of the isotope, the medium was removed, the cells washed with ice-cold Tris-buffered saline, collected, rewashed, spun down and stored at -70°C. Other normal or transformed cell lines were grown to near confluency, starved of phosphate, and pulse-labelled for four hours in the same manner as the infected cells.

RNA was extracted by a hot phenol procedure. The cells were suspended in 27 volumes of a solution containing 4 x 10^{-3} M $MgCl_2$, 0.5% sodium naphthalene disulfonate, and 1.25 mg/ml. bentonite. 3 volumes of 25% sodium dodecyl sulfate and 30 volumes of Tris-buffered phenol (pH 7.2) containing 0.1% 8-hydroxyquinoline were added. The suspension was shaken vigorously in a 62°C water bath for 3-5 minutes, then quickly cooled in an ice-salt mixture. Following another cycle of heating, shaking and cooling, the mixture was centrifuged. The aqueous layer

was removed and subjected to further deproteinization by shaking in the cold with an equal volume of the same phenol. The final aqueous phase was brought to 0.1 M NaCl, and the RNA precipitated by adding two volumes of cold 100% ethanol. The RNA was dissolved in SSC/10 (0.015 M NaCl, 0.0015 NaCitrate) containing 0.005 M $MgCl_2$ and treated with 10 γ/ml. RNase-free DNase for 45 minutes at 37°C. The mixture was then shaken with phenol, and the aqueous phase passed through a 55 cm-high column of G-100 Sephadex equilibrated with SSC/10 to separate the RNA from products of DNase digestion. The leading radioactive peak (RNA) was collected, precipitated with ethanol, redissolved in 2xSSC and stored at -70°C. In some instances the material from the column was filtered through nitrocellulose filters (Schleicher and Schuell Co., type B-6) once or twice to help remove small amounts of denatured DNA. The P^{32} in such preparations was rendered over 99.9% TCA-soluble by alkali treatment (0.3N KOH, 20 hours, 30°C), and was insensitive to further attack by DNase. The concentration of RNA in these preparations varied from 50 to 200 γ/ml.

(c) Hybridization

The technique used here is similar to that described by Gillespie and Spiegelman (1965). To convert the closed circular form of polyoma DNA (component I) to a heat-denaturable form (component II), the DNA was incubated with pancreatic DNase (10^{-5} to 10^{-4} γ/ml.) at room temperature until roughly 90% of the preparation was present as component II (according to Vinograd et al., 1965). The reaction was stopped by

addition of versene, and the DNA dialyzed against SSC/10. The DNA was denatured at a concentration of 20-40 γ/ml. by heating in boiling water for 10 minutes followed by rapid cooling in an ice-salt mixture. One-fourth volume of 2.5 M KCl, 0.05 M Tris, pH 7.4, was then added. Appropriate amounts of the DNA were filtered under suction onto nitrocellulose filters pre-soaked for 5-10 minutes in 0.5 M KCl, 0.01 M Tris, pH 7.4. (90-100% of the H^3-polyoma DNA deposited on the filters was retained, and not more than 5% of the initially retained counts was lost in the subsequent incubations and washings.) The filters were dried at room temperature for 1 hour, then in an oven at 80°C for 4-6 hours. In some cases, the filters were then placed in 2xSSC and incubated 10-14 hours at 60°C. This step was found to minimize the non-specific attachment or background of P^{32} in the subsequent incubation. For the formation of hybrids, the filters with DNA attached were placed singly in small chambers consisting of a plastic screw cap with fitting glass tube cut off just below the threads. The filter was placed inside the cap with the DNA-side exposed. The shortened glass tube was then screwed in place, serving as a well for the RNA solution. 0.4 cc of the RNA was added, and the open end of the tube was sealed off with vacuum grease and a cover slip. The chambers were then incubated in a 60°C water bath for 18-24 hours. At the end of the incubation, the filters were removed, washed thoroughly in 2xSSC, and incubated for 1 hour at 30°C in 1xSSC containing 20 γ/ml. RNase. The RNase was previously heated at 80°C for 15 minutes to inactivate traces of DNase. The filters were finally washed by passing through 80 cc of warm (56°C) 0.5 M KCl,

0.01 M Tris. After drying thoroughly, the filters were placed in plastic vials for counting in a Packard Tri-Carb Liquid Scintillation Counter. The channels were set for simultaneous counting of H^3 and P^{32}. Corrections for spillover between the channels were made whenever necessary. The approximate counting efficiencies were 15% for H^3 and 50% for P^{32} for unquenched samples.

3. Results

(a) Effects of Phosphate-deprivation on the virus growth cycle

The possibility that the low concentration of phosphate in the medium might prevent or alter the normal course of virus synthesis was examined by comparing the growth curves of virus under the normal and low phosphate conditions. Replicate cultures were harvested at various times after infection, frozen-thawed three times, and assayed for total virus by hemagglutination (Eddy, Rowe, Hartley, Stewart and Huebner, 1958). The yield at the end of the cycle was also assayed by plaque formation (Dulbecco & Freeman, 1959) to assure that infectious particles were present. The results are presented in Figure 1. It can be seen that both the time course of virus production and the final yield are the same for the two conditions.

(b) Detection of polyoma-specific RNA in infected mouse kidney cells

To detect the presence of viral-specific RNA in the RNA extracted from infected cells, samples of the separate pulse-labelled RNA preparations from each eight hour period from 0 to 32 hours after

infection were pooled. A constant amount of the pooled RNA was added to a series of filters containing increasing amounts of polyoma DNA. Two controls were included: RNA from pulse-labelled uninfected mouse cells added to polyoma DNA, and infected cell RNA added to E. coli DNA, which has a base composition close to that of polyoma DNA. The retention of E. coli DNA was determined by optical density measurements before and after filtration. Incubation was for 24 hours at 60°C. The results are shown in Figure 2; each point represents the average of two determinations. It can be seen that only in the case of infected cell RNA and polyoma DNA is there an increase in the amount of P^{32} counts bound with increasing DNA on the filters. The controls show that the major factor in the rise of this curve cannot be due either to the presence of an RNA in uninfected mouse cells which is homologous to polyoma DNA, or to a non-specific sticking to DNA by the infected cell RNA.

The kinetics of formation of the hybrid at 60°C is shown in Figure 3. Replicate hybrid chambers were set up with filters containing approximately 1.2γ of polyoma DNA and a constant amount of infected cell RNA. Duplicates were removed at the times indicated, and the amount of hybrid determined as described. Chambers containing filters without DNA and the same amount of RNA were included as background controls. It appears that no further hybrid is formed after about 16 hours, and the maximum amount of hybrid remains constant for up to 30 hours of incubation. In all subsequent experiments, the incubation time was between 18 and 24 hours.

(c) Appearance of virus-specific RNA during the growth cycle

Information concerning the relative amounts of virus-specific RNA at different times after infection was obtained through the following experiments. Samples of the pulse-labelled RNA from each successive eight hour period from 0 to 32 hours were diluted forty-fold from the initial concentration used above (part b), and incubated with filters containing increasing amounts of polyoma DNA in an attempt to "exhaust" the specific RNA in each of the RNA samples. The results are plotted as the fraction of the input counts of P^{32}-RNA hybridized versus the amount of polyoma DNA (Figure 4). The attainment of a plateau in such an experiment would indicate exhaustion, the level of the plateau giving directly the fraction of the labelled material capable of hybridizing to polyoma DNA. This would represent the actual virus-specific fraction if all of the virus-specific RNA present was capable of hybridizing (i.e., if there was no appreciably secondary structure in the RNA to prevent hybridization).

In the cases of the early periods, 0-8 and 8-16 hours, as the amount of polyoma DNA is increased, a small but increasing fraction of the RNA is bound above the background value (binding in the absence of DNA), and no plateau is reached at the DNA levels employed. In the later periods, 16-24 and 24-32 hours, the relative amounts of virus-specific RNA are much greater, and plateaus are reached with less than 1 γ of DNA (total RNA concentrations, approximately 5γ/ml). The RNA of the latest period appears to be richest in specific RNA, although even in this case less than one per cent of the labelled material hybridizes to virus DNA.

To verify that the RNA in the plateau region is actually depleted of virus-specific RNA and to rule out the possibility that the plateau is due to some limitation in the availability of the DNA for hybridization, the "exhausted" RNA was incubated a second time with a new filter carrying polyoma DNA. The amount of P^{32} bound in the second incubation was slightly above background, indicating that most, if not all, of the specific fraction was removed in the first incubation.

No direct comparison between the early and late periods in terms of the relative amounts of specific RNA can be made from these experiments due to the inability to exhaust the early period RNAs. Nevertheless, it appears that a sharp increase in production of virus-specific RNA occurs sometime after 16 hours.

The converse type of experiment, in which increasing amounts of RNA are added to a constant amount of DNA, was performed using the same RNA preparations. Since the background is not strictly linear with the amount of RNA added, separate background controls were included for each input RNA concentration; these background values were then subtracted from the counts bound with the same concentration of RNA in the presence of DNA. The results are plotted in Figure 5 as the cpm RNA hybridized per 0.1γ DNA versus the cpm incubated. The actual amount of DNA was around .07γ.

In principle, two kinds of information could be obtained from such an experiment. First, the initial slopes, in the region of DNA excess, are a measure of the relative amounts of virus-specific RNA, thus providing a common basis for comparing the RNAs from all periods. The

consistency of this method with that of "exhaustion" can be seen by comparing results obtained by the two methods with the late period RNAs: the ratio of the plateaus in Figure 4b is in good agreement with the ratio of the initial slopes in Figure 5b for the same RNAs, roughly 2:1 in each case. Secondly, the saturation of a low amount of DNA with RNA of any given period would allow the estimation of the fraction of the DNA involved in hybridization with the RNA of that period. It would appear from the results shown in Figure 5 that the first type of information could be obtained reliably from measurements of the initial slopes; the second type of information could not be obtained for any but the latest period RNA which seems to give a saturation of the DNA.

Experiments of this type were therefore repeated, using new preparations of RNA with successive 4 hour pulses from 4 to 32 hours after infection. RNA from each period was added in two concentrations to filters containing $0.5 \pm .04\gamma$ DNA. The relative amount of virus-specific RNA was claculated for each period by dividing the increase in counts of P^{32} hybridized by the increase in counts of P^{32} incubated for the two concentrations. These values, plotted against the time after infection, show the relative rate of synthesis of virus-specific RNA during the virus growth cycle (Figure 6). The uptake of C^{14} - thymidine into TCA-insoluble material by infected mouse kidney cultures is plotted for comparison. The increase in the fraction of hybridizable RNA after 16 hours is undoubtedly due in part to an increase in absolute amount of virus-specific RNA at these times; however, unknown factors,

such as changes in the rate of incorporation of P^{32} into other RNA (see below; since the overall rate of incorporation of P^{32} into RNA also increases during the late period, this factor seems somewhat unlikely), and changes in the specific activity of the phosphate pool with time, do no allow the exact determination of the absolute rate of virus-specific RNA synthesis.

Attempts to saturate a lower amount of DNA (roughly .07γ) with the 12-16 hour and 24-28 hour RNAs are shown in Figure 7. No saturation with the early RNA is observed when the maximum practical amount is added. The late RNA, however, appears to reach a saturation value of approximately 1.2×10^4 cpm per 0.1γ DNA. Assuming the RNA has the same specific activity as the medium (corrected for decay), this value corresponds to the binding to roughly 25-35% of a single viral DNA strand.

(d) Hybridization of infected and uninfected cell RNA with mouse DNA

Pulse-labelled RNAs from infected (16-24 and 24-32 hours, pooled) and uninfected cells were incubated with filters containing no DNA, 5γ of H^3-polyoma DNA, or 5γ of H^3-mouse DNA. The results are shown in Figure 8. As expected, only the infected cell RNA forms a significant amount of hybrid with the viral DNA. Both RNAs, however, hybridize with mouse DNA, the infected cell RNA to a greater extent than the uninfected.

(e) Detection of virus-specific RNA in transformed cell lines

Pulse-labelled RNAs from a series of normal and transformed cell lines were incubated with filters containing viral DNA in order to test for the presence of virus-specific RNA. All polyoma-transformed lines were tested for the presence of virus and were found to be virus-free (limit of detection: one plaque forming unit per 10^6 cells). Descriptions of the cell lines and results are given in Table 1. Results with some of the lines are presented graphically in Figure 9. Small but significant fractions of the labelled RNA from transformed lines hybridize with polyoma DNA. Virus-specific RNA can be detected in cells of three different species transformed *in vitro* by polyoma; no such RNA is found in either normal, SV-40 transformed or spontaneously malignant cells.

4. Discussion

(a) Virus-specific RNA in infected mouse kidney cells

A fraction of the P^{32} pulse-labelled RNA extracted from mouse kidney cells infected by polyoma virus is capable of hybridizing specifically with polyoma DNA. The size of this fraction depends on the time during the virus growth cycle at which the P^{32} is given. The curve showing the size of the virus-specific fraction as a function of time follows fairly closely the uptake of C^{14}-thymidine into DNA in the infected cultures (Figure 6). Since part of the DNA synthesized

after infection is known to be mouse DNA, a better comparison would be the time of appearance of infectious viral DNA (Dulbecco, Hartwell, and Vogt, 1965). DNA infectivity begins to appear around 22-24 hours and increases rapidly in parallel with the increase of virus-specific RNA. These results would suggest that progeny viral DNA acts as template for RNA synthesis.

From the saturation experiment with 24-28 hour pulse-labelled RNA (Figure 7b; Results, part c) it would appear that not all of the virus DNA is involved in hybridization with the late RNA; however, the calculation involves the untested assumption that all of the DNA on the filter is available for hybridization. The present results, therefore, give no clear indication as to the specificity of transcription of DNA sites during the course of virus development. It is hoped that such information can be obtained from further experiments, now in progress, involving competition between the early and late RNAs.

(b) Effect of infection on the ability of pulse-labelled RNA to hybridize with mouse DNA

During the period of maximum synthesis of virus-specific RNA (24-28 hours), not more than one per cent of the labelled RNA can hybridize to polyoma DNA. Even allowing for the fact that only half (roughly) of the cells in the culture are infected, it would appear that infection by polyoma does not block the uptake of P^{32} into cellular RNA. In fact, comparison of the abilities of infected and uninfected cell RNA to hybridize with cellular DNA shows a stimulation during

infection (Figure 8). These results could represent a "cross hybridization" between virus-specific RNA and mouse DNA, which would be predicted from the partial homology observed between synthetic polyoma RNA and mouse DNA (Winocour, 1965). On the other hand, they could indicate the actual stimulation of host cell RNA synthesis by the virus; this would be in keeping with observations made on the same system (Dulbecco et al., 1965) of a stimulation (or derepression) of mouse DNA synthesis. Evidence of a stimulation of cellular RNA synthesis has also been obtained in polyoma-infected mouse embryo fibroblasts (Benjamin, unpublished results).

(c) Presence of virus-specific RNA in transformed cells

The results presented here demonstrate that polyoma-transformed cells synthesize a rapidly labelled RNA which can hybridize to polyoma DNA. This finding is of particular interest in considering the role of the virus in causing transformation. The persistence of virus-specific RNA in these virus-free cells strongly suggests that the viral genome also persists, at least in part - possibly integrated with the DNA of the host. It would seem reasonable to suppose further that the virus-specific RNA functions in vivo as a messenger RNA; whether the corresponding protein would be required for maintenance of the transformed state is still a matter of speculation.

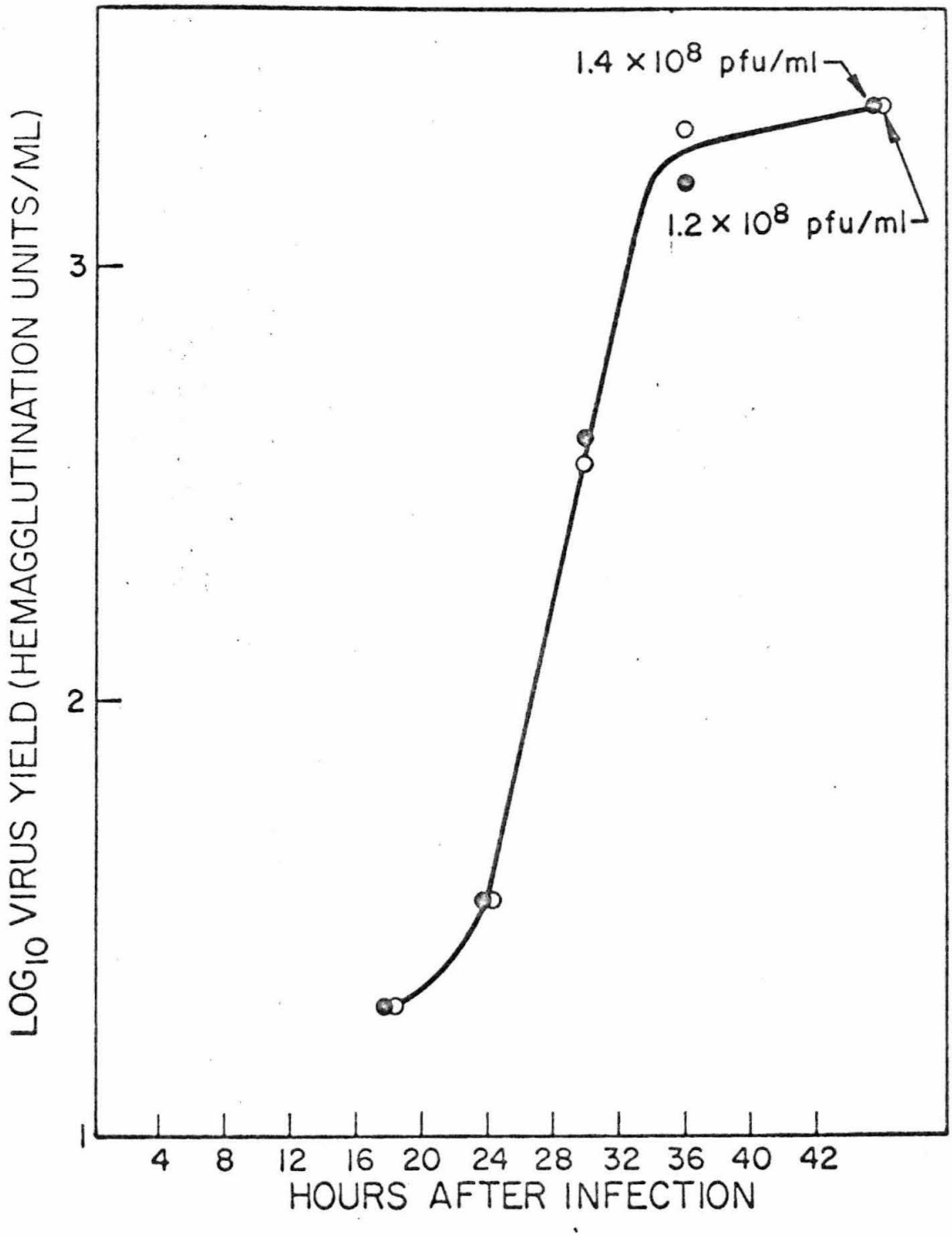

Fig. 1 - Growth curves of virus under normal (●) and low (○) phosphate conditions.

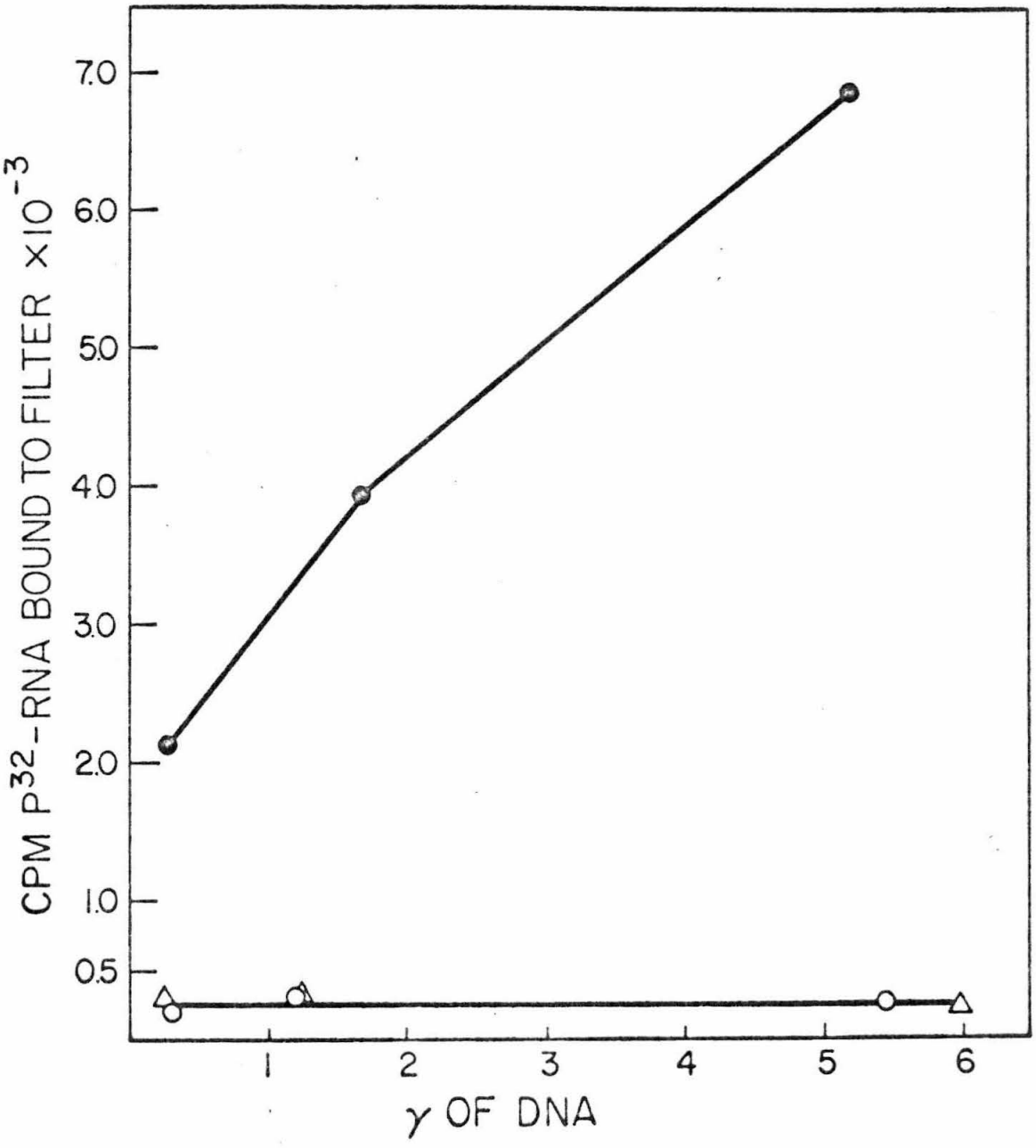

Fig. 2 - Test for virus-specific RNA in infected mouse kidney cells. ● - infected cell RNA, polyoma DNA; ○ - uninfected cell RNA, polyoma DNA; △ - infected cell RNA, E. coli DNA.

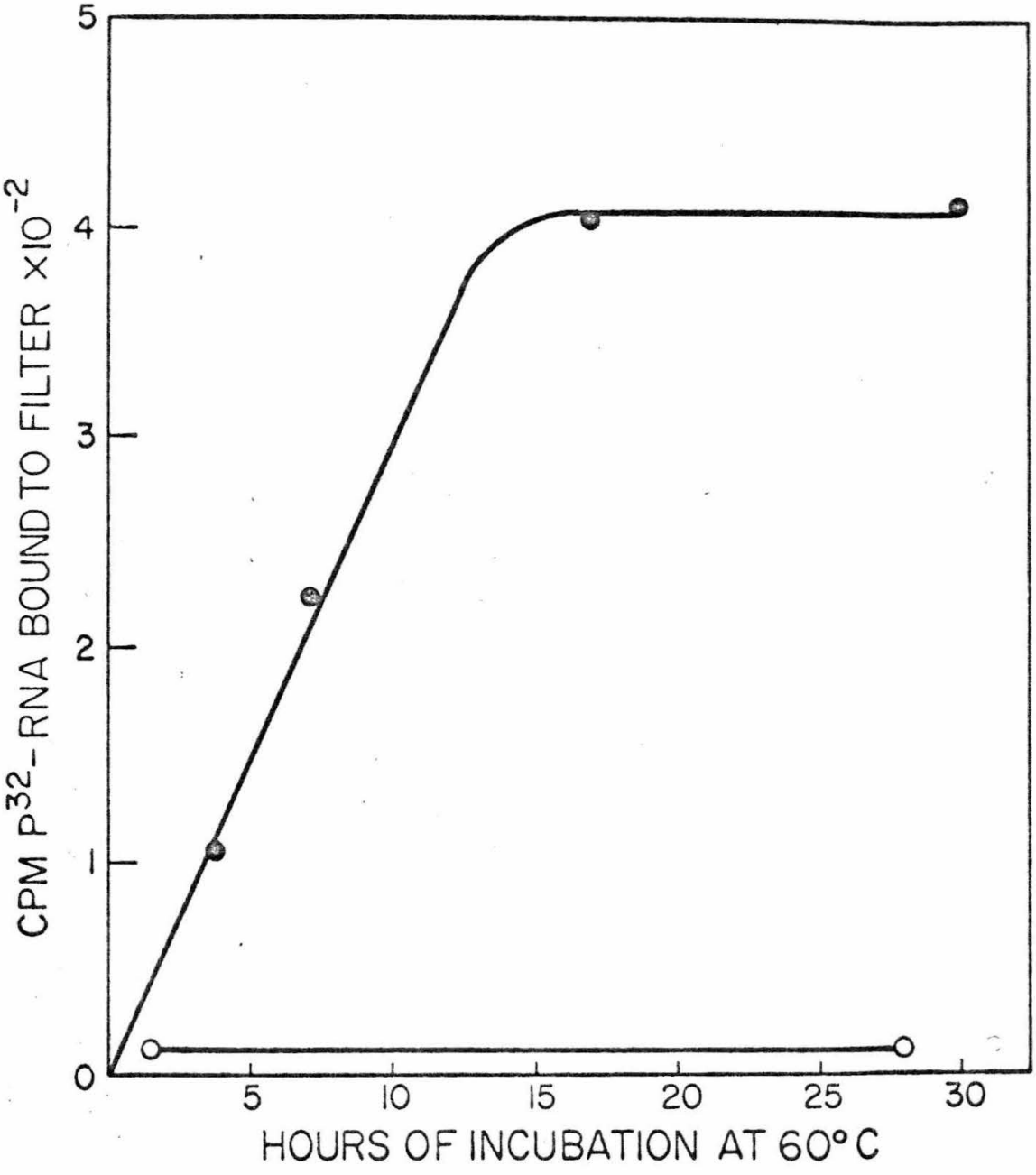

Fig. 3 - Kinetics of hybrid formation at 60°C. ● - hybrid, ○ - background.

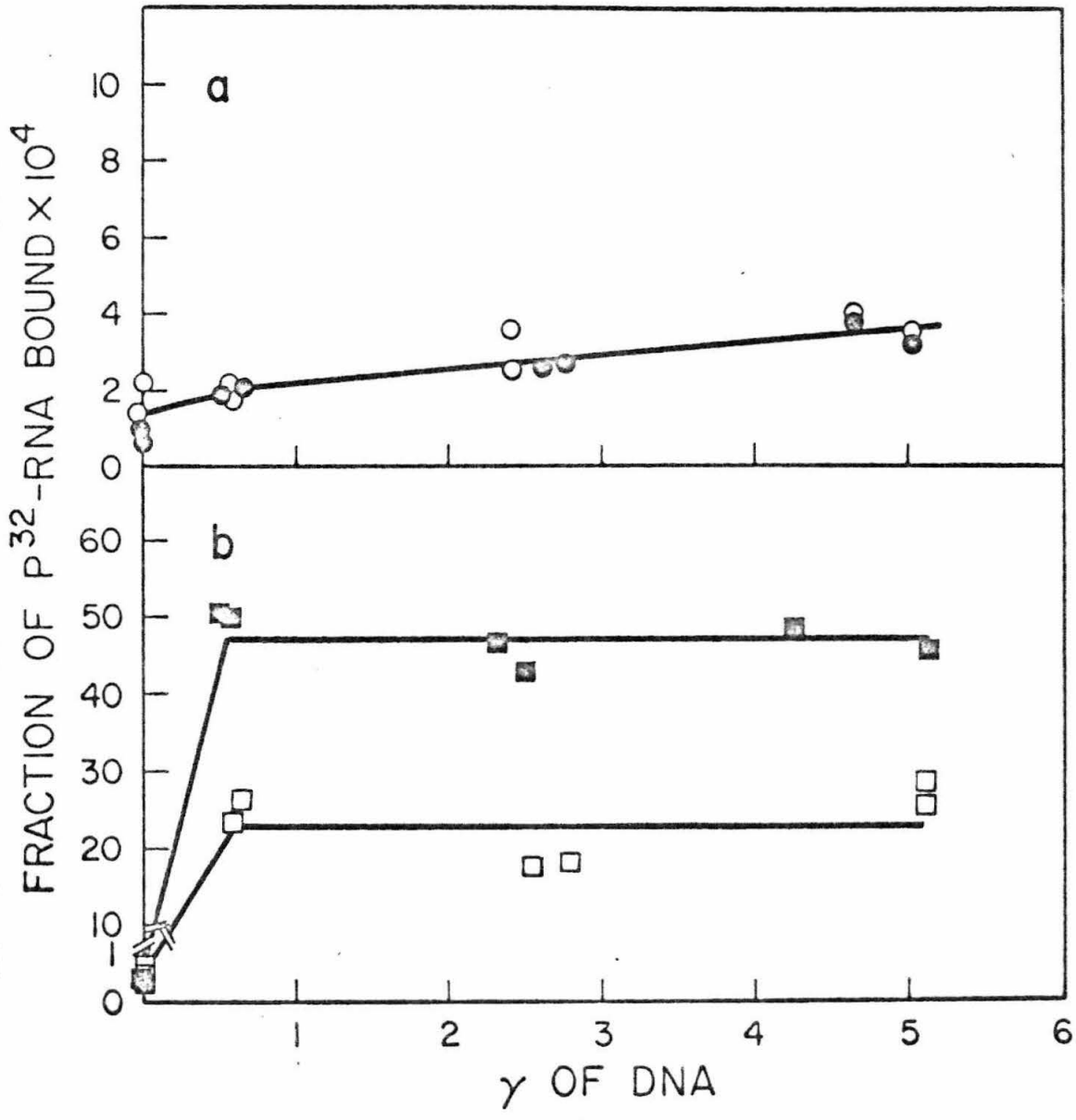

Fig. 4 - Attempts to exhaust virus-specific RNA from RNA of different periods. a) ○ - 0-8 hours, ● - 8-16 hours; b) □ - 16-24 hours, ■ - 24-32 hours.

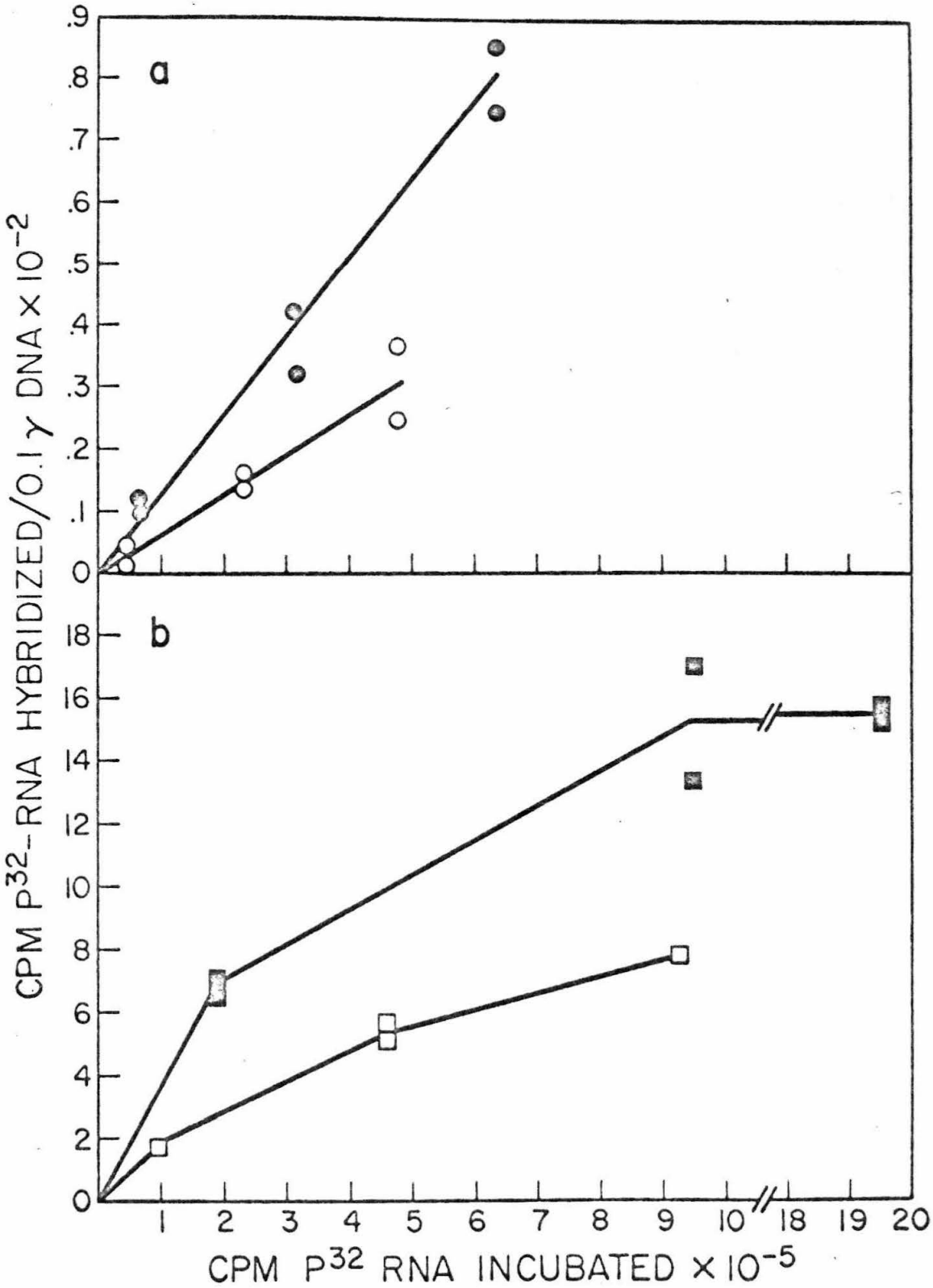

Fig. 5 - Attempts to saturate viral DNA with RNA of different periods. a) ○ - 0-8 hours, ● - 8-16 hours; b) □ - 16-24 hours, ■ - 24-32 hours.

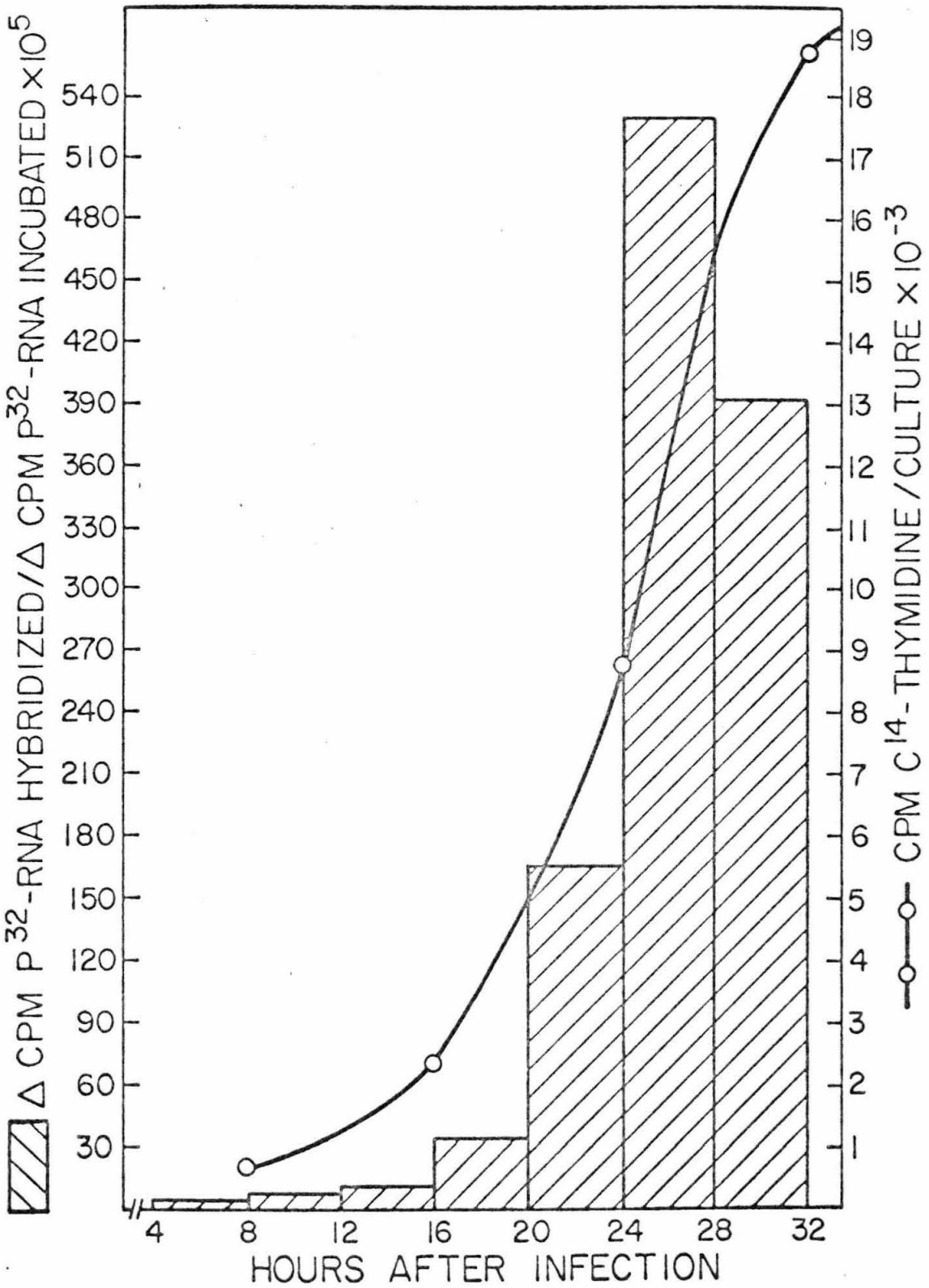

Fig. 6 - Relative amounts of virus-specific RNA at different times after infection.

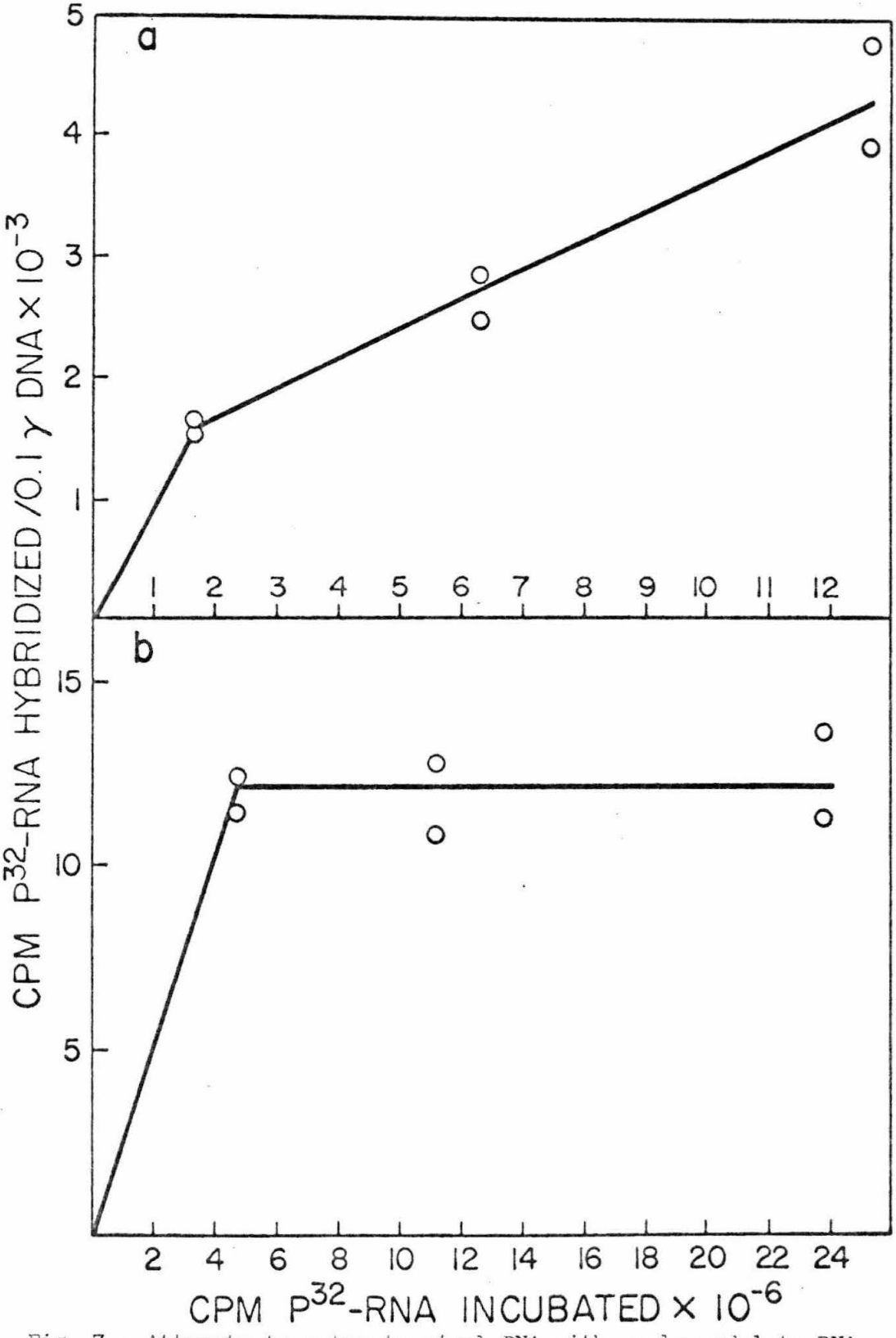

Fig. 7 - Attempts to saturate viral DNA with early and late RNA. a) 12-16 hour RNA, b) 24-28 hour RNA.

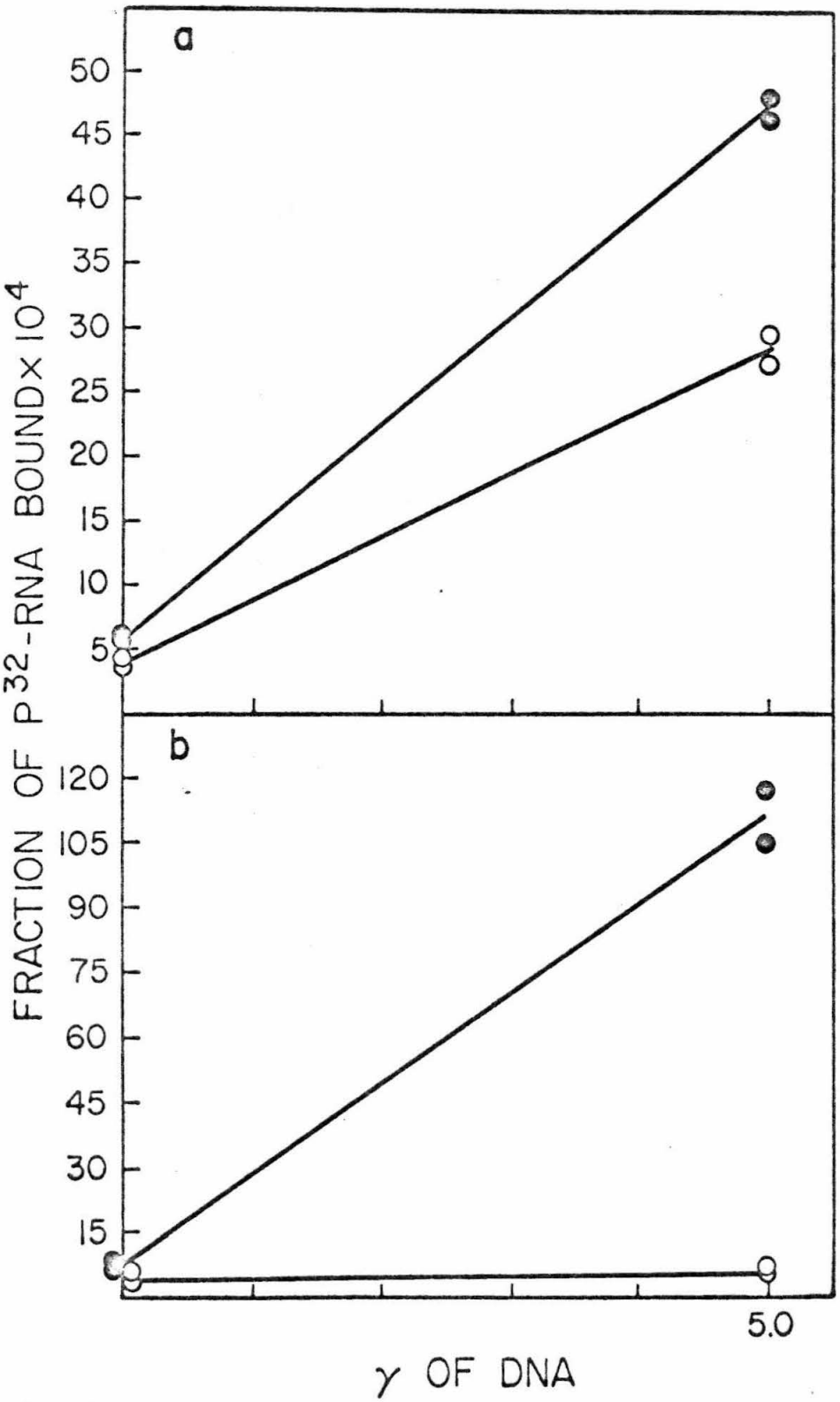

Fig. 8 - Hybridization of infected (●) and uninfected (○) cell RNA with mouse DNA (a) or polyoma DNA (b).

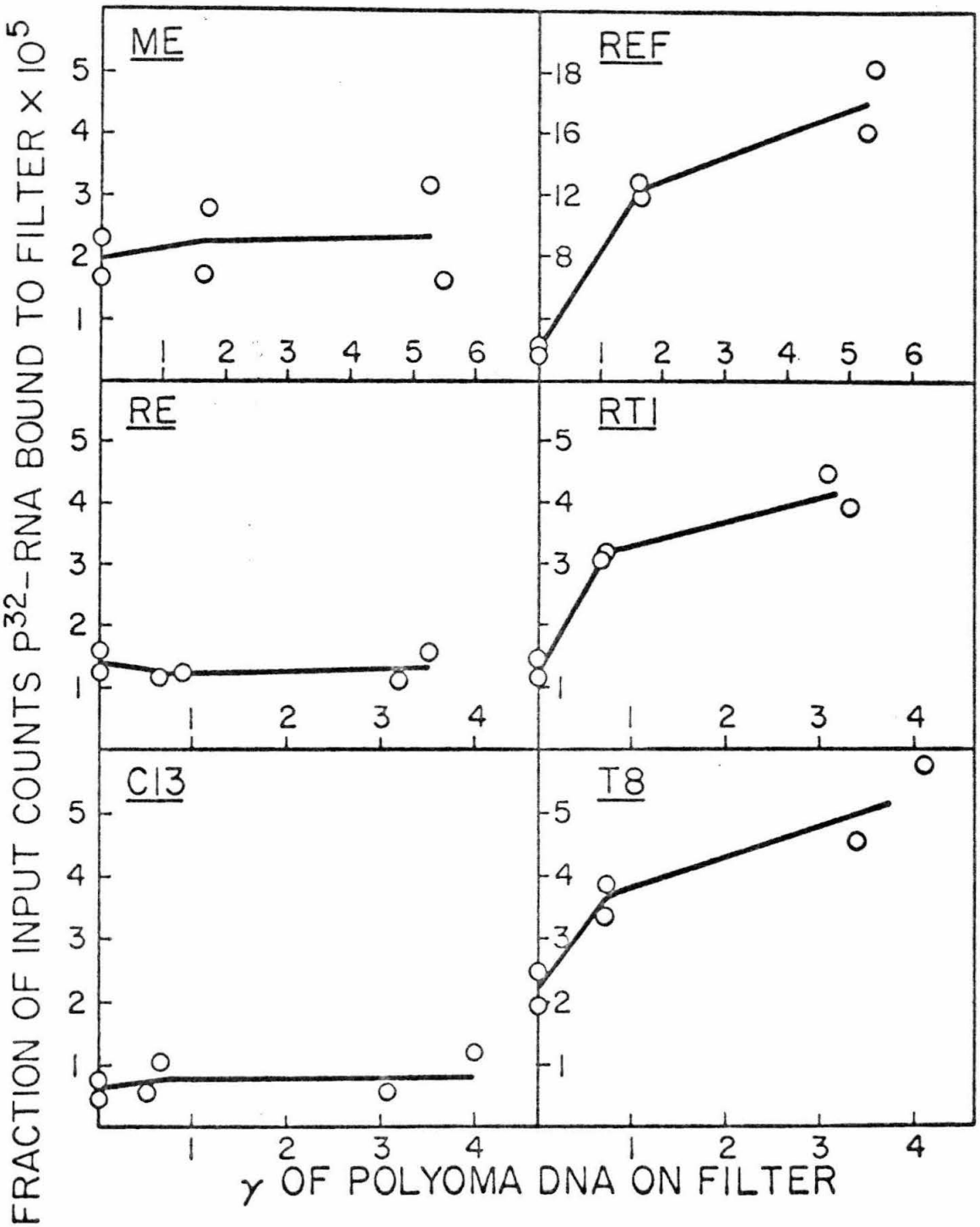

Fig. 9 - Test for virus-specific RNA in normal and transformed cells. (see Table 1).

Table 1. - Test for Virus-specific RNA (P^{32}) in Various Cell Lines

Cell Line	Origin and Description	Fraction* of P^{32} Bound to Filter x 10^5	
		- DNA	+ DNA (γ)
A) Mouse:			
ME	normal embryo secondary cultures	2.0	2.3 (5.3)
3T3	normal fibroblast (Todaro & Green, 1963)	4.0	2.8 (1.5)
Py-3T3-1	Py transformed derivative of 3T3 (Todaro & Green, 1965)	3.3	9.5 (1.8)
Py-3T3-11	similar to Py-3T3-1	3.1	17.6 (1.6)
SV-3T3-47	SV-40 transformed derivative of 3T3 (Todaro & Green, 1965)	1.4	0.9 (1.7)
SVPy-3T3-11	SV-40, Py doubly transformed (Todaro, Habel & Green, 1965)	2.1	19.2 (1.6)
3T3-Py-1	Py transformed derivative of 3T3 isolated in agar (Benjamin, unpubl.)	2.4	7.5 (1.6)
3T3-Py-6	Py transformed, similar to 3T3-Py-1	1.1	6.8 (1.7)
B) Hamster:			
C13	BHK, baby hamster kidney (Stoker & Abel, 1962)	0.6	0.8 (3.6)
Ha 651	"spontaneous" malignant derivative of C13, (see Bases, 1963)	4.2	3.4 (1.6)
Ha 442	similar to Ha 651	3.5	2.7 (1.6)
T1	Py transformed derivative of C13, isolated in agar (see Macpherson & Montagnier, 1964)	2.8	11.1 (1.6)
T8	Py transformed, similar to T1	2.1	5.1 (1.6)
S1	non-transformed, spontaneous mutant of C13 that grows in agar	3.8	2.5 (1.6)
C) Rat:			
RE	normal rat embryo fibroblast	1.4	1.3 (3.3)
RT1	Py transformed derivative of RE, isolated in agar	1.3	4.2 (3.2)
RT2	Py transformed, similar to RT1	2.2	10.8 (1.3)
RT4	Py transformed, similar to RT1	1.0	3.2 (1.3)
REF	Py transformed "focal" line, derivative of RE	2.0	17.2 (5.4)
RS2	non-transformed, spontaneous mutant of RE that grows in agar	4.8	8.3 (1.3)

*Average of two determinations

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REFERENCES

Axelrod, D., Bolton, E.T. & Habel, K. (1964). Science, 146, 1466.

Bases, R. (1963). Canc. Res. 24 (2), 1216.

Black, P.H., Rowe, W.B., Turner, H.C. & Huebner, R.J. (1963). Proc. Nat. Acad. Sci., Wash. 50, 1148.

Defendi, V. (1963). Proc. Soc. Exptl. Biol. Med. 113, 12.

Dulbecco, R. & Freeman, G. (1959). Virology, 8, 396.

Dulbecco, R., Hartwell, L.H. & Vogt, M. (1965). Proc. Nat. Acad. Sci., Wash. 53, 403.

Eddy, B.E., Rowe, W.B., Hartley, J.W., Stewart, S.E. & Huebner, R.J. (1958). Virology, 6, 290.

Gillespie, D. & Spiegelman S. (1965). J. Mol. Biol. 12, 829

Habel, K. & Eddy, B.E. (1963). Proc. Soc. Exptl. Biol. Med. 113, 1.

Habel, K. (1965). Virology, 25, 55.

Huebner, R.J., Rowe, W.P., Turner, H.C. & Lane, W.T. (1963). Proc. Nat. Acad. Sci., Wash. 50, 379.

Koch, M.A. & Sabin, A.B. (1963). Proc. Soc. Exptl. Biol. Med. 113, 4.

Macpherson, I. & Montagnier, L. (1964). Virology 23, 291.

Murakami, W. (1963). Science 142, 56.

Sjogren, H.O., Hellstrom, I. & Klein, G. (1961). Cancer Research 21, 329.

Stoker, M. & Abel, P. (1962). Cold Spring Harbor Symp. Quant. Biol. 27, 375.

Ting, R. (1964). Virology, 24, 227.

Todaro, G.J. & Green, H. (1963). J. Cell. Biol. 17, 299.

Todaro, G.J. & Green, H. (1965). Science 147, 513.

Todaro, G.J., Habel, K. & Green, H. (1965). Virology 27, 179.

Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. & Laipis, P. (1965). Proc. Nat. Acad. Sci., Wash. 53, 1104.

Vogt, M. & Dulbecco, R. (1962). Virology, 16, 41.

Weil, R. (1961). Virology, 14, 46.

Winocour, E. (1963). Virology, 19, 158.

RECAPITULATION AND CONCLUSIONS

A. Brief Recapitulation of Results

1. In Part I no radiobiological evidence for "temperateness" of Py was found. During the course of this investigation, a two-slope survival curve was observed for the inactivation of Py by ultraviolet light. Analysis of some factors affecting the shape of the curve led to the postulation of a host cell repair mechanism in mouse cells.

2. In Part II it has been shown that slightly less than half of the "hits" (UV, X-ray, nitrous acid, or P^{32} decay) to the plaque-forming ability of Py leave the transforming ability intact.

3. In Part III methods were developed that allowed the detection in both productively infected and virus-free transformed cells of a rapidly labelled RNA which is capable of hybridizing with the viral DNA.

B. General Conclusions

From the results of Part II it is concluded that not all of the genes required for virus multiplication are required for obtaining transformation. Considering the small amount of DNA in the virus, it would seem that transformation is due to the action of not more than a few genes. Thus, the action of Py in causing transformation may be described as "partial" or "defective" in the sense of leading to incomplete virus development. This situation may or may not be closely parallel to that with RSV. While only a defective state of the RSV

genome is known, two possibilities may be envisaged for Py: 1) expression of the basically non-defective genome is partially repressed by some unknown mechanism during the transformation interaction, and 2) transformation is due to a fraction of particles carrying defective genomes. In the latter case, the alteration would have to be of a subtle nature not involving gross changes in the DNA structure. The second alternative seems unlikely in view of the high multiplicities of infectious virus used to cause transformation.

The persistence of viral genes in the transformed cell directing the synthesis of the virus-specific RNA is strongly suggested from the results of Part III. The possibility that the fraction of the genome relevant to transformation (Part II) is homologous, at least in part, to the virus-specific RNA present in the transformed cell should be considered. On the basis of several lines of evidence, the relevant part should be the fraction corresponding to "early" functions. Thus, it would be predicted that the virus-specific RNA of transformed cells should compete with "early" cytolytic RNA. This prediction may be tested experimentally.

The presence of viral function(s) in transformed cells allows a further prediction to be made which can be tested by extending the inactivation studies in the following manner: When a UV-irradiated virus particle infects a transformed cell, the presence of virus functions in the cell should lead to a by-pass of some of the UV damages; thus a reduction of the target size of the plaque-forming ability of Py should be observed when the survival is measured on transformed, rather than normal cells.

Finally, in light of the hybrid results, the question of the relationship of the new antigens of transformed cells and viral genes becomes more meaningful. This question might be approached by dissociating the virus-specific RNA of transformed cells from the viral DNA and testing its ability to direct the synthesis of protein *in vitro*. The relationship of the product protein to the antigens might then be tested by one of several immunological methods.