VIRUS CARCINOGENESIS

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1. Introduction

The title of this discussion, "Virus carcinogenesis," was purposely made general because there are at present differences of opinion both with respect to (1) the nature of the tissue reaction designated as cancer, or neoplasia, and (2) the mechanism through which viruses are thought to act to bring about this peculiar reaction [1]. It is not the purpose here to go into these differences of opinion at length, or to discriminate among them in an attempt to present a final picture of virus carcinogenesis at this time. Rather, the purpose will be to present certain representative quantitative data, together with descriptions of the conditions under which they were derived, so that so far as possible the thinking of mathematicians and the tools of mathematical statistics may be brought to bear on the basic problem of discerning ultimate mechanisms in this area of carcinogenesis.

Since many viruses are known which produce tissue reactions or diseases other than cancer, and since biologically active agents of various types other than viruses are capable of inducing cancer, it is obvious that *virus carcinogenesis* cannot be considered in an isolated fashion, without some reference to the biological responses to agents of these other types. Preliminary discussions will therefore be given of selected quantitative biological data which illustrate the types of results obtained with chemical carcinogenic agents and with viruses other than those which produce cancer.

2. Subcutaneous injections of polycyclic hydrocarbons (chemical carcinogens)

In another paper of this Symposium, Dr. Blum deals with the induction of cancer by ultraviolet light, as well as by repeated doses of polycyclic hydrocarbons applied to the skin of experimental animals. The data to be reiterated here were obtained following the subcutaneous injection of single doses of three different chemical carcinogens (methylcholanthrene, dibenzanthracene, and benzpyrene) into respective groups of inbred mice [2]. In each case subgroups of animals were injected with decreasing doses in a geometric series, prepared by making serial twofold dilutions of a starting concentration which was determined by the upper limit of solubility of the chemical in the oily medium (tricaprylin) used as diluent and injecting vehicle.

As in laboratory experimentation in general, the nature of data acquired will be influenced to some degree by the particular laboratory techniques used in carrying out the experiments. This is illustrated most strikingly by the differences that will become apparent on comparison of the results obtained following single subcutaneous injections of chemical carcinogens with those obtained following repeated applications to the skin.

Even within technical groups the results may vary according to the immediate methods by which experimentalists make their observations. For example, the biological response to chemical carcinogens is greatly protracted in time, and many months may elapse between injection of the chemical and the appearance of cancer in the experimental animals. In order to quantitate the *response time* component of the data, the observer examines his animals at scheduled intervals and records a given cancer as having "appeared" either at the time of the first positive examination, or at the mid-point of the interval between the last negative and the first positive examination. But the length of the interexamination interval and the manner in which the experimentalist makes his physical examinations can influence the results for the following reason: oily solutions, both natural and foreign, induce a tissue reaction by the host when injected subcutaneously, whether or not they contain dissolved carcinogenic chemicals. The reaction is similar to that provoked by "foreign bodies" in general, and represents a walling off of the "foreign body" by proliferation and migration of fibrocytes of the host to form a capsule of fibrous tissue. In the case of oily foreign substances such as those used for dissolving chemical carcinogens the capsule may surround most of the inoculum to form a single, large, palpable cyst; or, it may be multilocular and composed of numerous minute, nonpalpable or even microscopic cysts. In the routine palpations for *detection* of tumors the pressure exerted is sufficient to rupture the delicate cyst walls, since one of the criteria for gross diagnosis of a cancerous nodule is its degree of firmness or its failure to be deformed by considerable pressure. The length of the interexamination interval will therefore determine the frequency of rupture of the oily cysts, as well as the ease with which they may be ruptured, that is, the longer the interval, the thicker the cyst wall and the greater its resistance to rupture. Thus, in the local response to chemical carcinogens dissolved in oil, there is a continuing struggle between the host in its defensive reaction against a foreign substance and the experimenter who periodically breaks down this reaction.

The effect of keeping the inoculum dispersed by frequent rupture of the cysts is to maintain larger numbers of host cells under continuous exposure to the maximum concentration of hydrocarbon in the inoculum. On the other hand, the same procedure will favor decay in the concentration of chemical through metabolic activities of cells and through diffusion and elimination via the blood stream and excretory organs. These factors might be expected to influence both the time to response and the final incidence of positive responses, at least at the lower effective dose levels.

Other factors also affect the actual magnitudes of the parameters of response.

Many of those associated with the host, such as genetic constitution, environmental conditions, diet, sex, and age at time of treatment, can be controlled by experimental design. Others, such as "natural" mortality during the prolonged observation periods associated with the study of carcinogenic chemicals, cannot be controlled experimentally, but methods are available for statistical control and interpretation of the data.

A final source of variation which might be mentioned at this point is one which has not yet been taken into consideration by experimental biologists, but which definitely should be, as has been emphasized by Neyman (personal communication). It is the variation in size at time of detection of the small cancerous nodules. For example, for theoretical studies it is necessary to know not only the average number of cells required for sensory perception of a lesion, but also the distributions of sizes perceived by different observers as well as by the same observer at different times. It is to be hoped that future investigations will supply this, now missing, information.

The data presented in figures 1 to 4 are from a collaborative study with Shimkin published in 1943 [2]. They represent the results obtained following single injections of carcinogenic hydrocarbons into the subcutaneous tissues of the right axilla of C3H male mice. Examinations by palpation were made every 4th or 5th day beginning 4 weeks after injection. A deliberate attempt was made at each examination to break up any cyst which had formed, thus dispersing the inoculum. The cancers were recorded as having "appeared" at the time they were first detected on gross examination.

Figure 1 illustrates the development of tumors with time, following the injection of various doses of methylcholanthrene. It shows the correlation with dose, below a certain dose level, of both the median response time and the variation of individual response times about their medians. The results were essentially the same for the other two hydrocarbons (dibenzanthracene and benzpyrene) except for the specific values of the respective response times in relation to dose.

The interpretation of this correlation between response time and dose has been dealt with by others, notably by Iversen and Arley [3] in their theoretical mathematical discussion, and therefore will not be gone into in detail here. However, the major components of the lag phenomenon might be reiterated. The first is the "true latent period," or the time required for an appropriate "hit," or interaction between hydrocarbon molecules and sensitive cell components. Iversen and Arley [3] have estimated the nature of the probability distribution of this event with time.

The second major component of response time is the interval required for growth of a colony of cells from one or more initially transformed cells, to a size which is detectable by the method being employed, for example, palpation or visual inspection. Thus the observable response is an "amplification" [4], [5] of the initial biological effect, brought about through growth and proliferation of cellular elements. The probabilistic problem associated with this phase of the reaction is therefore one in population statistics, involving birth-and-death proc-

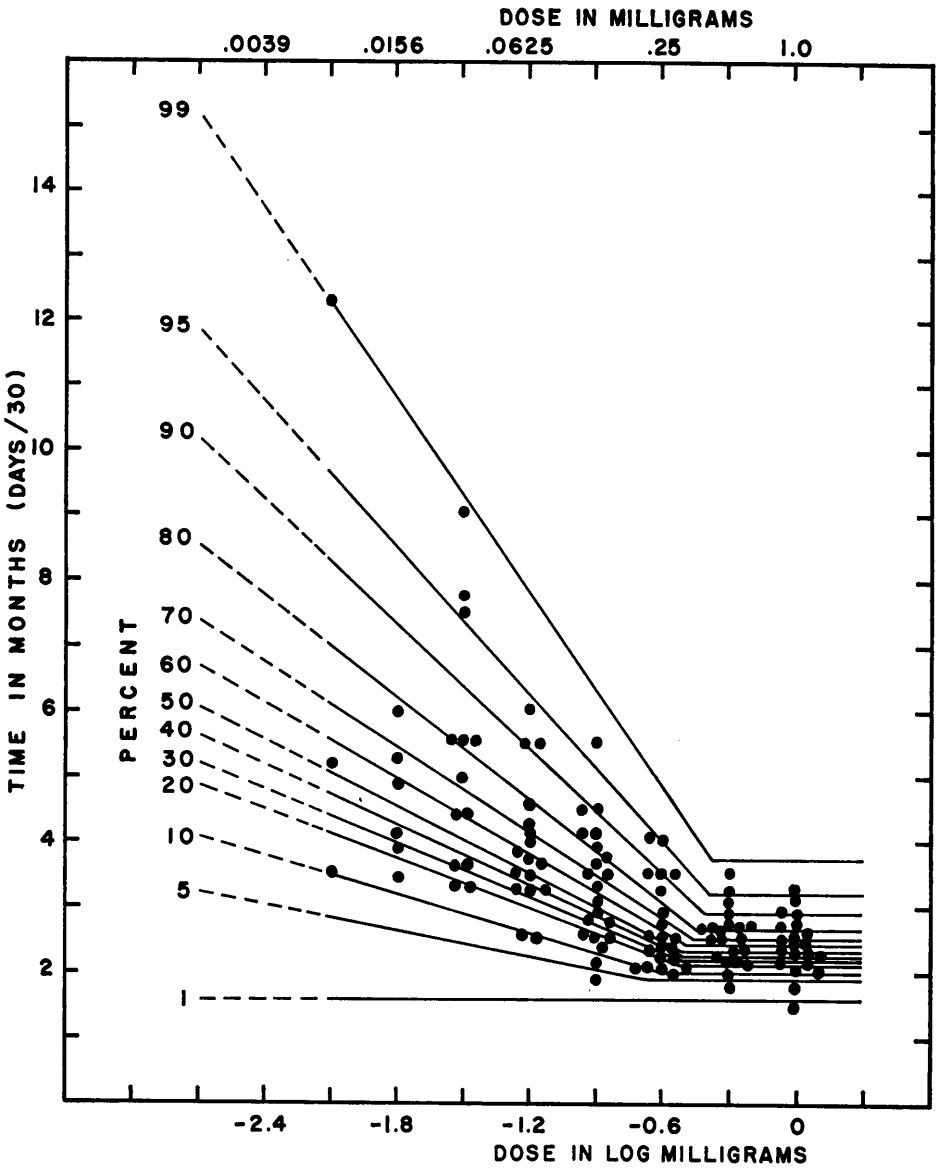

FIGURE 1

Time to development of cancers in mice
treated with successive doses of methylcholanthrene.
(Data of Bryan and Shimkin [2], figure 1.)

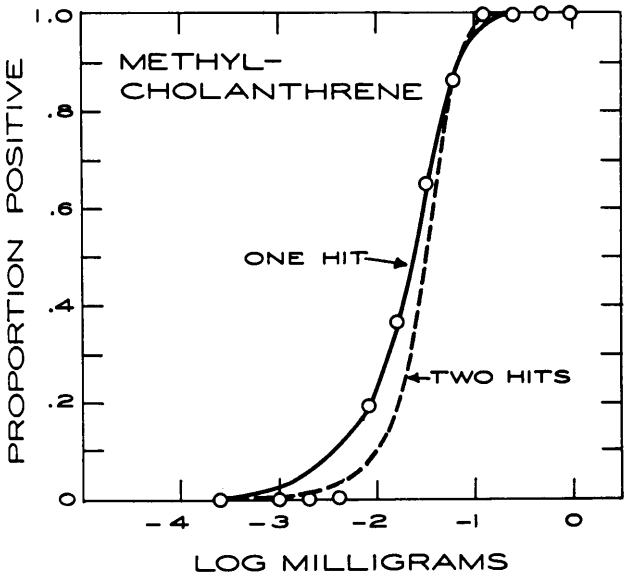

FIGURE 2

Final incidence of cancers in mice treated with successive doses of methylcholanthrene. (Data of Bryan and Shimkin [2], table 1.)

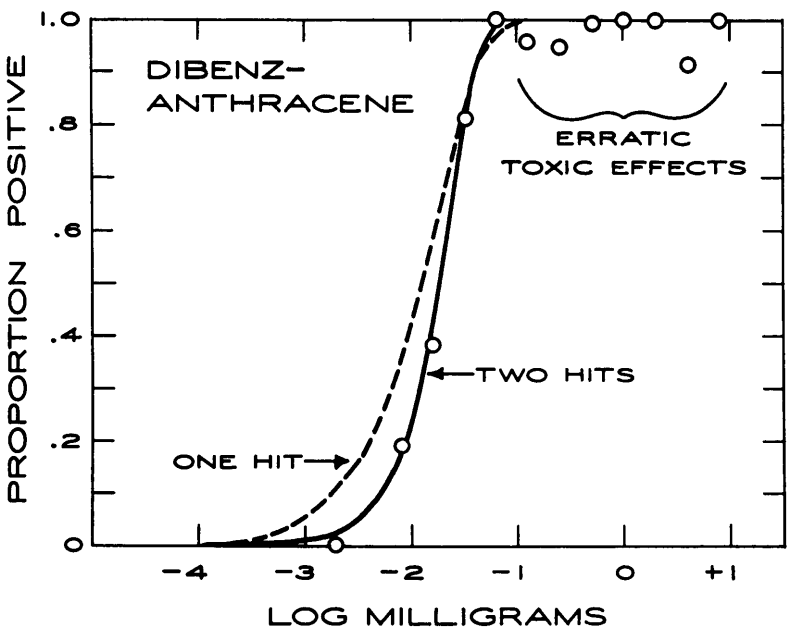

FIGURE 3

Final incidence of cancers in mice treated with successive doses of dibenzanthracene. (Data of Bryan and Shimkin [2], table 4.)

esses of cells [6], [7]. (See also the paper by Tucker, in this Symposium.)

The comparative data of primary interest in this discussion are the cancer-incidence results at "infinite" time, shown for different dose levels in figures 2, 3, and 4, for methylcholanthrene, dibenzanthracene, and benzpyrene, respectively. From 16 to 22 mice were included initially in all dose groups except the two lowest dose groups for each hydrocarbon, which contained 2 and 4 times this number, respectively. After correction for noncancer mortality, the significant numbers ranged from 15 to 22 for the higher doses and from 32 to 70 for the

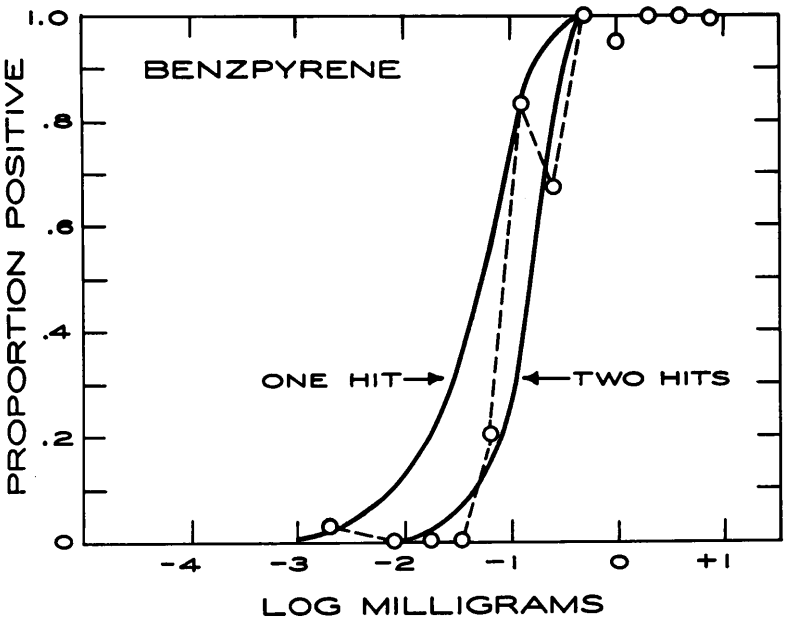

FIGURE 4

Final incidence of cancers in mice treated with successive doses of benzpyrene. (Data of Bryan and Shimkin [2], table 7.)

two lowest doses. By "infinite" time is meant the practical limit beyond which the frequency of cancers no longer increases appreciably with time. It is this final dose-response curve that will be compared among carcinogenic chemicals, viral agents other than those which induce cancer, and cancer-inducing viruses themselves.

It will be noted that the results shown in figure 2 for methylcholanthrene give an excellent fit to a theoretical "one-hit" curve, whereas those shown in figure 3, for dibenzanthracene, give an equally good fit to a theoretical "two-hit" curve. In neither case were the deviations from the theoretical curves statisti-

cally significant. As pointed out in the original report of these studies [2], the results obtained with benzpyrene were heterogeneous, and deviated significantly from the fitted normal curve used at that time as a mathematical model for analysis. Figure 4 shows that the results with this hydrocarbon fail also to fit either a one-hit or a two-hit curve. The theoretical curves shown were dropped from the point of the lowest dose which yielded an observed response of essentially 100 per cent. It may or may not be of significance that the scattered and heterogeneous incidence results for this hydrocarbon, between 100 and zero per cent, fall closely about either one or the other of these two theoretical curves.

Results obtained in living animals are subject to many variable host factors during the prolonged experimental period extending from youth and young adulthood, to physiological old age. These factors include not only the drastic physiological and endocrinological changes with maturity and old age, but also, contact with disease-causing organisms and intercurrent "natural" diseases to which the species is subject. Knowledge that cancer response data are thus complicated by both intrinsic and extrinsic influences requires that caution be used in basing theoretical interpretations on limited amounts of data acquired in studies on living animals.

Since the basic events associated with carcinogenesis are undoubtedly intracellular, it is evident that information on mechanisms might be more efficiently achieved with in vitro systems, involving the treatment in tissue culture of known numbers of cells with known doses of carcinogenic chemicals. However, there are at present no criteria by which cancer cells in general can be definitely distinguished from normal cells growing in tissue culture, and strictly in vitro studies on mechanisms of chemical carcinogenesis are not yet feasible.

3. Viruses that destroy host cells

3.1. *Responses of rabbits to intradermal inoculations of vaccinia virus.* One of the viruses most widely investigated with respect to quantitative dose-response relationships is the rapidly acting vaccinia virus, which produces localized necrotic lesions within a few days when injected intradermally into experimental animals. Vaccinia is the live vaccine virus used for vaccination against smallpox. Figure 5 shows the incidence of positive inoculation sites on rabbits obtained by Parker [8] with a strain of the virus which had been highly adapted to the rabbit by many serial passages in this animal. Each of the doses of the twofold dilution series was inoculated in the amount of 0.25 ml. at 5 or 6 different sites on each of 11 rabbits. (Estimated from other information given by Parker [8]. The actual number was not stated.) The total number of site inoculations varied from 69 to 76 for the different doses. Since the lesions develop within 3 to 5 days after virus inoculation, even with the lowest effective doses, such factors as "natural" mortality and changing age and physiological status of the host do not complicate the results at "infinite" time (5 days) as

they do in studies on chemical carcinogens. Furthermore, the aqueous solutions used as vehicle for inoculating viruses do not provoke complicating "foreign body" reactions, as do the oils used in work with carcinogenic chemicals.

As is indicated by the close grouping of the points about the solid line of figure 5, the results give an excellent fit to a theoretical one-hit curve. This finding led Parker [8] to conclude that the phenomenon responsible for the one-hit curve was the chance presence or absence of one-or-more virus particles in the unit volumes used for inoculation. It was even suggested [8], [9] that such "titration curves" obtained in living animals might provide a means for esti-

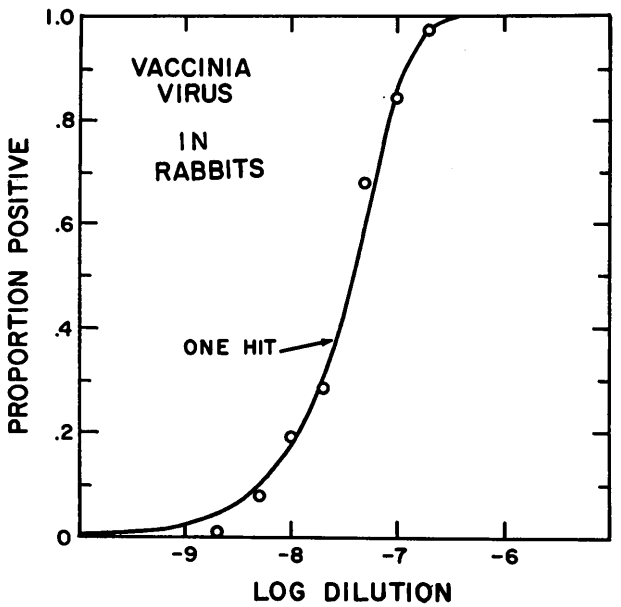

FIGURE 5

Final incidence of positive reaction sites on rabbits, inoculated with successive doses of vaccinia virus. (Data of Parker [8], experiment 1.)

mating absolute numbers of virus particles in unknown virus preparations. However, this has not proven feasible in actual practice, for reasons that have been discussed in detail elsewhere [4], [10], [11]. In brief, they are: (1) deviations of the results from theoretical one-hit curves, and (2) variations in the numbers of physical particles of virus which correspond to the unit biological response (that is, 63 per cent positive and 37 per cent negative) in animals of different genetic types, or in animals of the same type at different times or under different conditions [4].

When living animals are used as the biological test unit a close fit to the theoretical one-hit curve is the exception rather than the rule. Figure 6 shows the type of deviation most commonly observed with viruses of infectious diseases.

It is a breaking away of the higher percentage incidence results with the stronger doses as the curve approaches 100 per cent. These data were also published by Parker [8] for another rabbit-adapted strain of vaccinia virus. As pointed out by Armitage and Spicer [11] this is precisely the result to be expected on mathematical grounds, if there is significant biological variation among individual animals (or sites on animals) with respect to degree of susceptibility to a virus; that is, unless the animal variation is very great, or very heterogeneous, appreciable variations from the theoretical curve will occur only at the higher incidence levels.

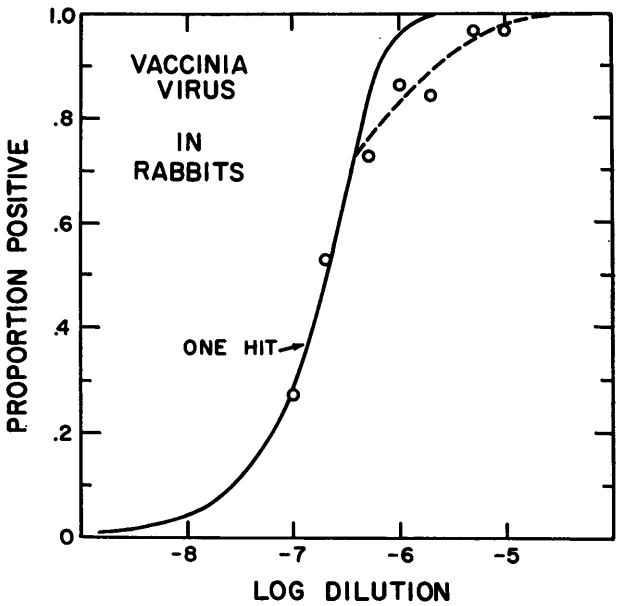

FIGURE 6

Final incidence of positive reaction sites on rabbits, inoculated with successive doses of vaccinia virus. (Data of Parker [8], experiment 4.)

It should be mentioned that the observable response to vaccinia virus is also an "amplified" reaction [4], [5] since the initial response to one or a few particles of virus could not be detected by visual inspection of the sites of skin inoculation. Although there is some cellular proliferation initially, the "amplification" results primarily from virus replication accompanied by destruction of cells and spread of the virus to new cells, that is, from a chain type of necrotizing reaction which continues until gross lesions become visible.

3.2. *Responses of cells in tissue culture to an adenovirus.* Results with an adenovirus in a tissue culture system were chosen for illustrating two points simultaneously: first, the advantages of in vitro test units over intact animal hosts for studying basic intracellular biological mechanisms; and second, the

protraction in time of the final effects of a cell-destroying virus that is comparable to, though less exaggerated than, the time lag associated with the actions of cancer-inducing viruses.

Figure 7 shows the percentage of tissue culture units exhibiting cell destruction at successive times following inoculation of groups of cultures with the serial doses of adenovirus indicated along the abscissa. The data are those of Pereira and Kelly [12]; the figure is reproduced from a previous publication by the author [4] which dealt with these same data. It will be noted that the left and middle curves, representing observations at 20 days and 9 days, respectively,

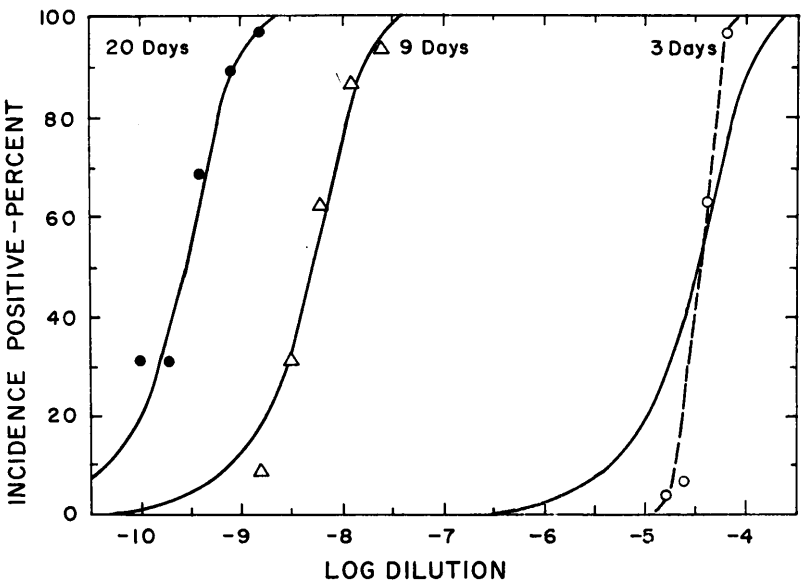

FIGURE 7

Incidence of tissue culture units showing cell destruction at successive times following inoculation with serial doses of adenovirus.
(Data of Pereira and Kelly [12], reproduced from [4].)

give excellent fits to the observed results indicated by the plotted points. The solid curves are theoretical one-hit curves drawn through the observed 50 per cent effective dose in each case. As is illustrated by this experiment, when a single large lot of cell suspension is used for preparing multiple tissue culture units of a common series there is little, if any, significant variation among the susceptibilities of the individual units. Thus, contrary to the findings in animal systems, adherence to a one-hit curve is the rule, rather than the exception, when the biological units are preparations of cells growing in tissue culture. When an exception occurs, as in the extreme right curve of figure 7, there is usually some obvious complicating factor. In this case it was a nonviral toxic substance present in strong concentrations of the virus preparation (tissue cul-

ture fluid from a previous culture) used for inoculating the cells. In subsequent studies by Pereira [13] it was found that the toxic factor could be removed either by differential ultracentrifugation, or by digestion with the enzyme, trypsin, under conditions which did not harm the virus.

Figure 8 is a reproduction of one of the figures published by Pereira [13] in his additional study of adenovirus in a tissue culture system. It further illustrates

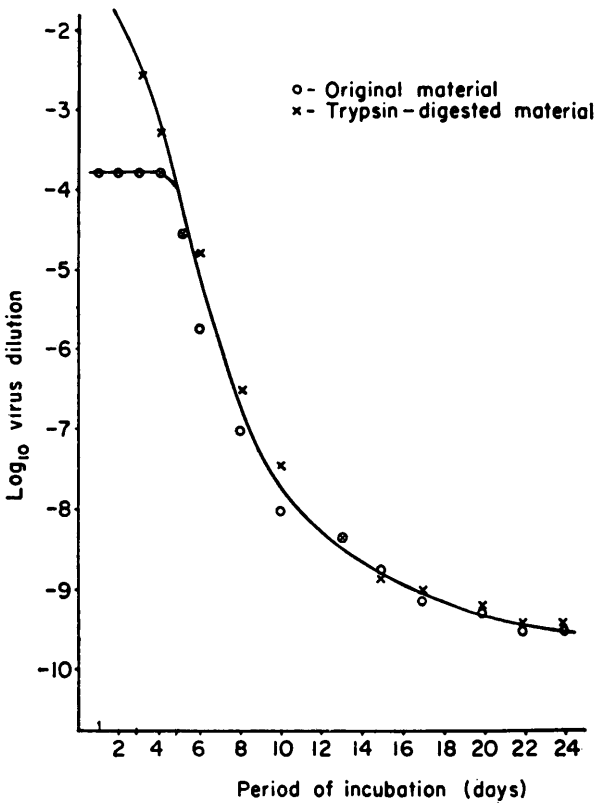

FIGURE 8

ED50 for cell-destroying effects determined at successive times following inoculation of tissue cultures with adenovirus.
(Data of Pereira [13], figure 1.)

the protraction in time of the cell-destroying effect in relation to dose of virus. The plotted data represent ED50 values estimated from individual curves such as those of figure 7, at successive times following inoculation of the cultures with doses of virus in a similar dilution series. The fitted curve of figure 8, with its limb corresponding to "trypsin-digested" virus material at the highest concentrations, was used as a basis for constructing the series of curves shown in figure 9. The latter represent the progression with time of the incidence curve in relation

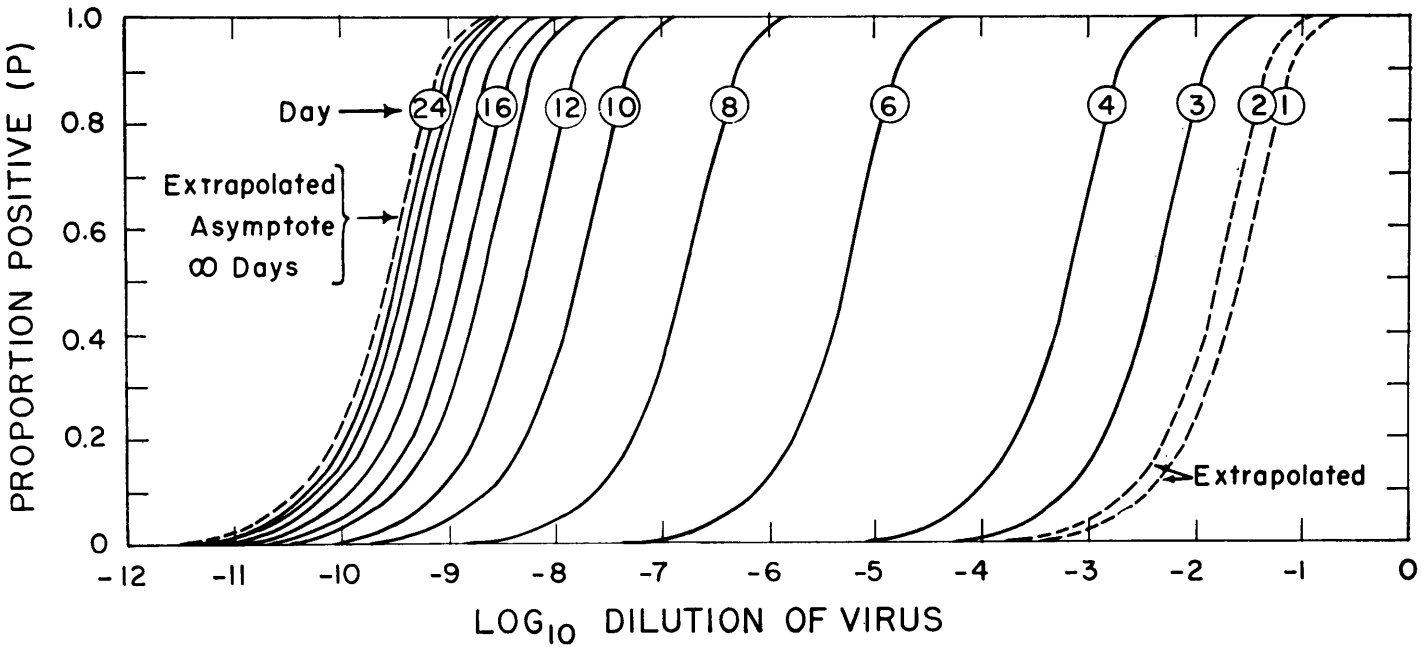

FIGURE 9

Estimated time-dose-response relationships for adenovirus in a tissue culture system. (Derived from data of Pereira [13].)

to dose of virus. Detailed data of the separate curves of this study were not published by Pereira, but the assumption has been made that they were consistently Poisson one-hit curves, as they were for the uncomplicated results at 9 days and 20 days in the earlier investigation (see figure 7). Figure 9, therefore, represents the *response plane* for the occurrence of positive responses (cell destruction) with time, in relation to infecting dose of virus. This more complete picture of the relationship derived from the observed trend of the ED50 values of figure 8 strengthens the original conclusion of Pereira and Kelly [12] that "the probability of virus-cell interactions is a function of time." This conclusion is consistent with all evidence available at the present time, and it undoubtedly is of fundamental importance in the interpretation of quantitative biological responses to viruses in general, including carcinogenic viruses.

Figure 9, or the general mathematical equations which would describe the response plane, may therefore be looked on as an ideal limit which is approached in the interactions of highly susceptible cells with relatively slowly acting agents of the adenovirus type [14]. Since the observable responses to even the most rapidly acting cancer viruses are similarly protracted in time, the general mathematical model may possibly be applicable, also, to cancer viruses in unraveling complex animal-host reactions to viruses which are slow-acting, or which produce "latent" infections. Therefore, it may be profitable to consider apparently bizarre observed results as representing deviations from ideal *response planes*, as Armitage [15] has done for *response curves* at "infinite" time, in an attempt to discover factors which can account for the nature of the observed deviations.

4. Cancer viruses

4.1. *Rous sarcoma virus.* The diversity of final tumor-incidence results obtained with carcinogenic viruses may be illustrated with selected data procured with one of them, the Rous sarcoma virus [16], in studies carried out at different times or under different controlled host conditions. The Rous sarcoma virus is specific for the connective-tissue cells of fowls. It produces highly malignant sarcomas in domestic chickens, which were the hosts employed in all of the experiments to be described herein. The laboratory procedures were also the same in all of the examples to be presented. Standardized samples of virus of known potency were diluted in tenfold steps and subgroups of 18 to 50 or more chickens were inoculated with each of the dilutions in a given series. The volume of the inoculum was constant at 0.2 ml., and all injections were made into the subcutaneous tissues of the left wing web. Routine examinations for the detection of tumors were made by visual inspection. The examinations were carried out at scheduled intervals on a reciprocal of time scale [17] over a period of about 35 days, which is the practical limit beyond which tumors do not appear with significant probability at sites of inoculation of this virus. Tumors were recorded as having "appeared" at the mid-point of the interval between the last negative and the first positive examination. Two different lots of frozen standardized

virus (CT559 and CT581) were involved in the series of studies ([18] to [21]) reproduced in figures 10 through 15, but both lots of virus had the same biological potency within limits of bioassay error (± 0.3 $\log_{10}$ units).

The experimental results shown in figures 10 through 15 were selected out of a large number of experiments to illustrate the different types of dose-response curves that have actually been observed. In the order in which they are arranged, they illustrate a progressive decrease in average susceptibility to the virus within breeds of chickens, as well as a progression in the degree of departure of the observed results from theoretical one-hit curves. Following the lead of

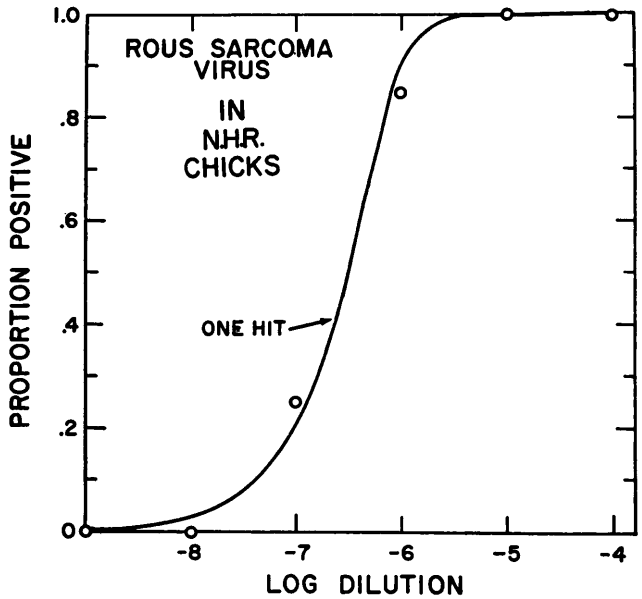

FIGURE 10

Final incidence of cancers in New Hampshire Red chickens inoculated with successive doses of Rous sarcoma virus. (Data of [19], table 7.)

Armitage [15] the deviations were considered as resulting from heterogeneous variations in host susceptibility, and the theoretical curves were fitted at the lowest doses to yield positive results, that is, cancers.

As illustrated in figure 10, some lots of chickens are fairly homogeneous and yield results which fit a theoretical one-hit curve. The chickens in this experiment were of the New Hampshire Red breed. They were derived from a commercial source which maintained its own, closed, breeding flock, but the animals were not closely inbred in the genetic sense. The virus used in this experiment was of lot CT581.

The results of figure 11 were obtained with the same lot of virus (CT581) and with chickens from the same flock (New Hampshire Red) as in the preceding

experiment. The breaking away in this instance of the observed results from the theoretical curve at the higher dose levels suggests that there was a significant amount of variation among the hosts in this experimental lot, comparable to that shown for vaccinia virus in figure 6.

The experiment depicted in figure 12 involved New Hampshire Red chickens from the same breeding flock, but virus of a different frozen lot (CT559) from that employed in the two preceding experiments. As already pointed out, the two different virus preparations had the same potency as determined by bioassay.

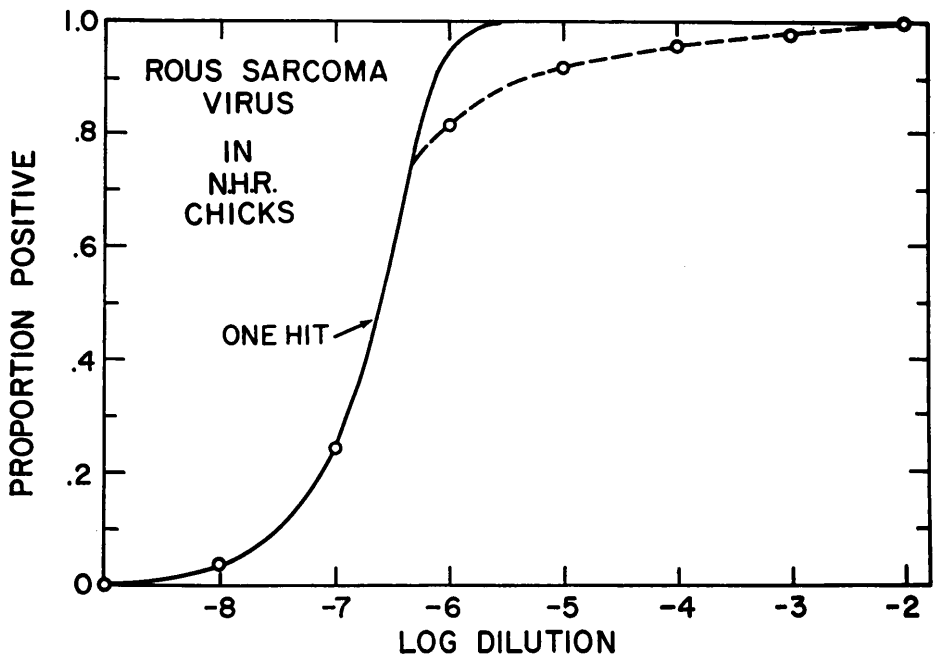

FIGURE 11

Final incidence of cancers in New Hampshire Red chickens inoculated with successive doses of Rous sarcoma virus. (Data of [19], table 8.)

Yet about 1.2 $\log_{10}$ units more virus were required in this instance to produce a 50 per cent response. The chickens of this experimental lot were therefore less susceptible to the virus, on an average, than those of the preceding experiments. That they also were more variable is indicated by the greater departure of the body of observed results from the theoretical curve.

The results of the remaining figures of this series, 13, 14, and 15, were obtained with samples of a common lot of virus (CT581), but involved three different genetically inbred lines of White Leghorn chickens. The latter had been developed by brother-to-sister matings over a period of about 20 years at the U.S. Department of Agriculture Foultry Research Laboratory in East Lansing, Michigan

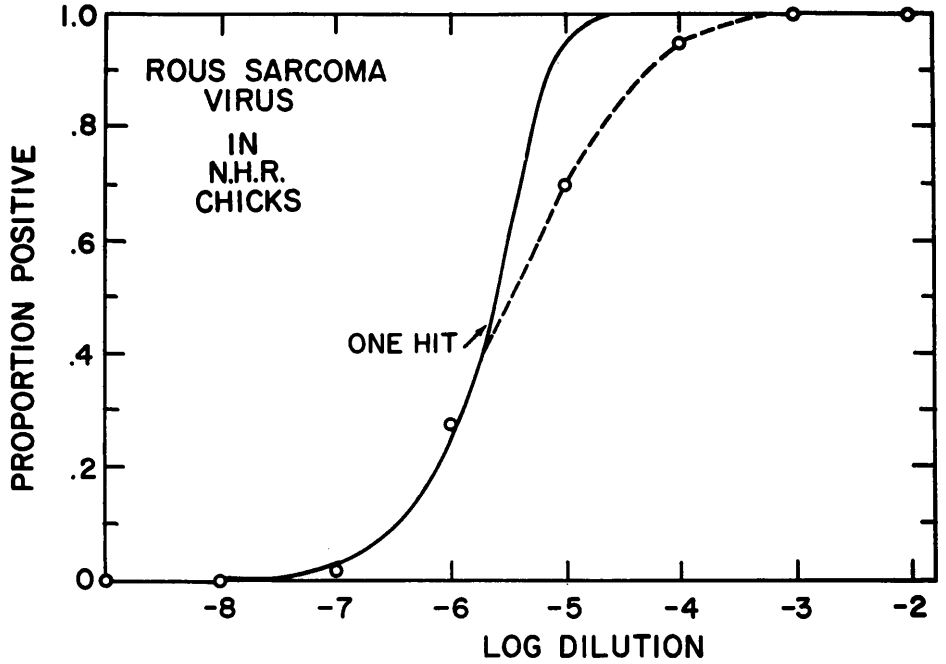

FIGURE 12

Final incidence of cancers in New Hampshire Red chickens inoculated with successive doses of Rous sarcoma virus. (Data of [18], table 1.)

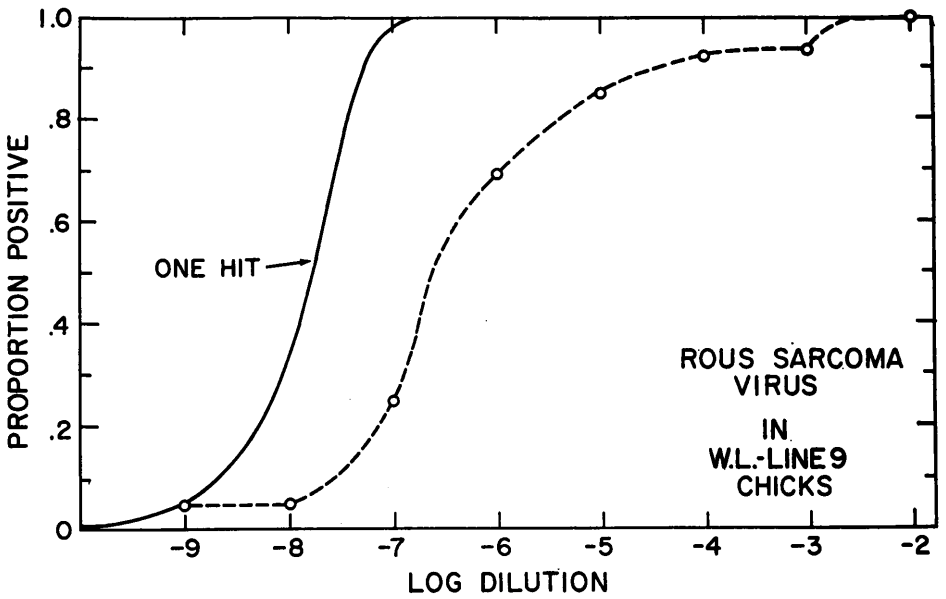

FIGURE 13

Final incidence of cancers in White Leghorn chickens of line 9 inoculated with successive doses of Rous sarcoma virus. (Data of Burmester et al. [21], table 7.)

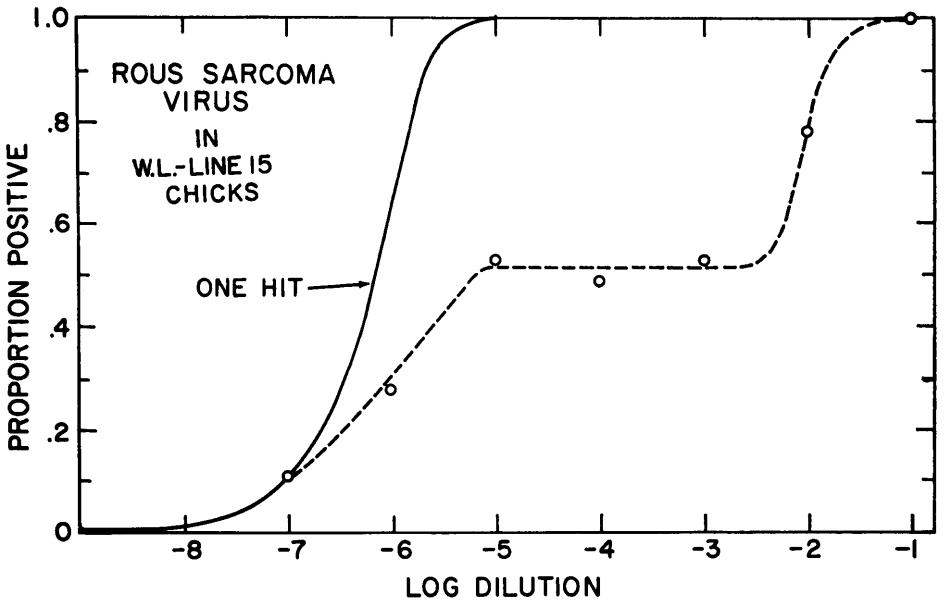

FIGURE 14

Final incidence of cancers in White Leghorn chickens
of line 15 inoculated with successive doses of Rous sarcoma virus.
(Data of Burmester et al. [21], table 7.)

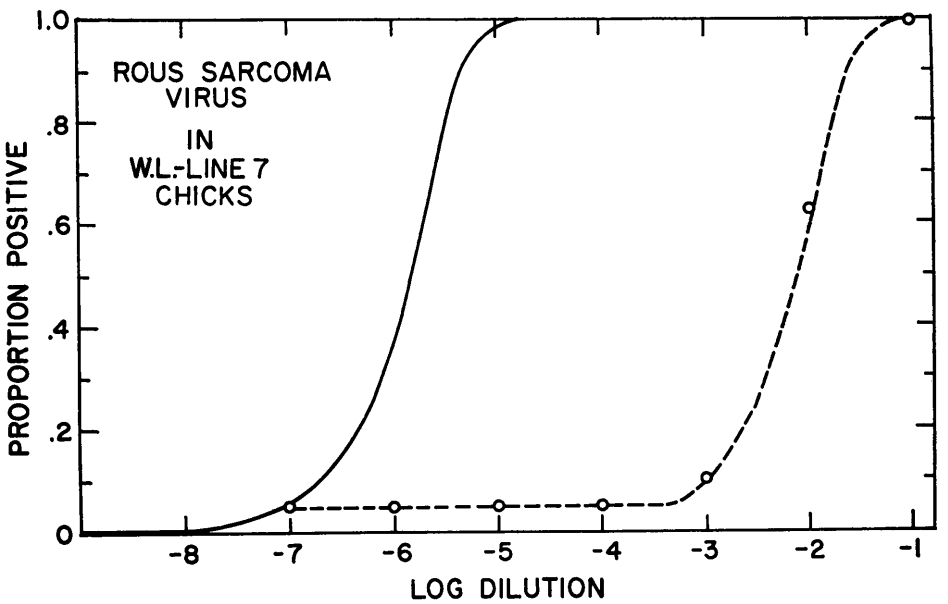

FIGURE 15

Final incidence of cancers in White Leghorn chickens
of line 7 inoculated with successive doses of Rous sarcoma virus.
(Data of Burmester et al. [21], table 6.)

[22], [23]. In developing the separate inbred lines selection had been made both toward (lines 9 and 15) and away from (line 7) *susceptibility* to the fowl *lymphomatosis* virus. At the time of the studies represented in these figures, the various lines were calculated to be only about 80 to 90 per cent inbred with respect to homogeneity of genetic material.

Preliminary studies by Burmester (see [21]) had indicated that White Leghorn chickens of the inbred lines that were most susceptible to lymphomatosis virus were also highly susceptible to the Rous sarcoma virus, and, conversely, that the inbred line least susceptible to lymphomatosis virus was also the least susceptible to Rous sarcoma virus. More comprehensive studies, in which the author collaborated, were carried out in 1956 for comparing the responses of 5 different inbred lines to Rous sarcoma virus. It was hoped that the close inbreeding over so many years would have resulted in: (1) experimental chicken populations which would be more homogeneous and therefore better suited for quantitative biological investigations than ordinary commercial populations; and (2) populations of highly different average susceptibilities, which would make possible controlled investigations on the host factors which determine susceptibility or resistance to Rous sarcoma virus.

Although the second desideratum was achieved so far as average susceptibilities were concerned, the inbred chicken populations did not appear to be suitable for quantitative investigations because of the heterogeneity of the group responses and the bizarre appearance of the dose-response curves (figures 13 to 15). These data were therefore placed on the shelf, so to speak, and their publication was not further contemplated until 1958, when Dr. Armitage examined them and suggested their interpretation on a basis of a "two-point" distribution concept, which he subsequently published [15]. The data on all 5 of the inbred lines which had been investigated were later published [21] in sufficient detail to permit others to examine them critically.

The results on 3 of the lines have been selected for illustrating, respectively, mixed populations in which: (a) the majority of the chickens were highly susceptible but some of them were relatively resistant (line 9, figure 13); (b) approximately one half of the chickens were highly susceptible and the other half relatively resistant (line 15, figure 14); and (c) most of the chickens were relatively resistant but a few of them were highly susceptible (line 7, figure 15). The broken lines of the figures were drawn by sight, with a two-point distribution bias, through the observed results indicated by the plotted points. The solid curve in each case represents the theoretical one-hit curve fitted to the lowest dose at which positive results were obtained. Since none of these experiments actually embraced a zero response at the lowest dose tested, it is possible that the displacement from the theoretical curves is even greater than has been indicated. However, the estimates of displacement are probably correct within a dosage factor of 10, since cancers have been observed only rarely with the -9 or lower $\log_{10}$ dilution of virus having this standardized potency, in numerous tests involving highly susceptible chickens of various breeds and strains. The experi-

ment represented in figure 13 includes one of these rare instances. In fact, this set of data, taken alone, is suggestive of three discrete susceptibility categories and a possible "three-point" distribution.

By assuming various two-point distributions having the same mean, but with different proportions of test units in the two relative-susceptibility categories, Armitage [15] has generated "dose-response" curves by mathematical procedures which come amazingly close to some of the "bizarre" observed results shown here. Taking into consideration the fact that a two-point distribution is an idealization used for a first approximation, and that some variation about the mean would be expected to occur within each of the susceptibility categories, it seems reasonable to expect that with adequate information on such distributions, mathematical theory might be expanded to account for even the most bizarre observed dose-response curves. In the statistical connotation of the term, "contaminated" populations appear to be the rule among test hosts available for investigations on cancer viruses. Biologists simply have to live with this fact, and do the best they can under the circumstances. Since genetic mutations are continuously taking place even among the most highly inbred animal populations, it seems unrealistic, biologically, to hope that eventually stable and ideally uniform animal populations will become available for cancer-virus research. A more realistic hope would appear to reside in the enlistment of mathematicians in greater numbers, and in the collaboration of mathematicians and biologists in the development of mathematical theory, and tools for coping with the complex experimental data as they actually exist today.

Yet to be explored is the value of response planes of the type shown in figure 9 for analyzing the reactions of living animal hosts to cancer viruses and other carcinogenic agents. Detailed data which would permit such analyses have been published for most of the experiments used in the foregoing illustrations. Figure 16 shows the time-dose-response plane for one of them, the homogeneous chicken group the final results on which were given in the curve of figure 10. Since no additional tumors appeared after the 20th day in this experiment, the latter curve, at "infinite" time (35 days), is identical with and superposable upon the 20-day curve of figure 16. It is seen that the observed results give a good fit to the theoretical one-hit curve (solid lines) at each of the times represented in the figures after the 6th day, that is, at 13, 20, and 35 days. This indicates that, as for adenovirus in a tissue culture system [12], the probability of interactions in this virus-animal system is also a function of time. The importance of this factor in virus infections of the "latent" type is obvious.

It has already been pointed out that strictly in vitro techniques are not yet available for the study of chemical carcinogenesis in tissue culture. This was true also for virus carcinogenesis until recently, when developments with two cancer viruses, one of them the Rous sarcoma virus [24], [25], [26] made possible the detection of what appears to be malignant transformation in vitro. It has also been demonstrated [25], [26] that successful infection of cells in vitro by Rous sarcoma virus is not synonymous with malignant transformation, and that

under certain conditions the cells may be successfully infected, so that they will produce more virus, but without manifesting the morphological changes characteristic of malignant transformation. Some other event, or set of physiological conditions, is therefore apparently necessary for the virus to bring about malig-

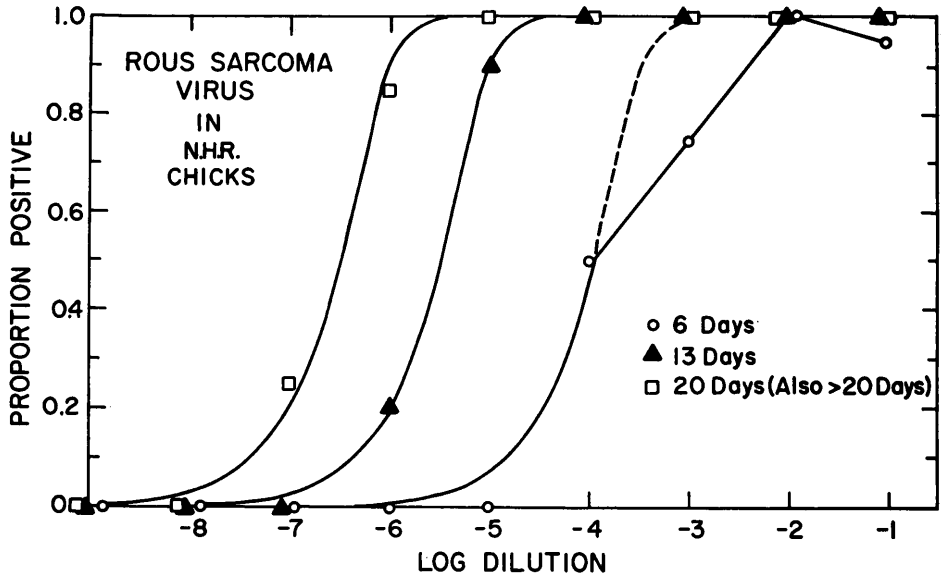

FIGURE 16

Incidence of cancers at successive times in New Hampshire Red chickens inoculated with serial doses of Rous sarcoma virus. (Data of [19], table 7.)

nant change in the cells. This is an important development because it now makes possible the study in an in vitro system, of one of the most fundamental characteristics of cancer viruses, namely, their ability to remain in a latent state in an infected host for relatively long periods of time without showing overt evidence of their presence.

5. Other tumor viruses

Except for the actual magnitudes of the time periods elapsing before development of the neoplastic responses, the final incidence curves obtained with other tumor viruses that have been investigated are comparable to those shown in the preceding section for the Rous sarcoma virus. The more general designation, *tumor viruses*, is used at this point rather than *cancer viruses*, in order to include the agent causing Shope rabbit papilloma [27], which is a benign neoplastic lesion (or tumor) rather than a malignant cancer. However, the benign lesions induced by this virus may eventually progress to the point of becoming malignant cancers.

Detailed time-dose-response data similar to those cited for Rous sarcoma virus have been published for the Shope rabbit papilloma virus [28], the fowl myeloblastosis virus [29], [30], and the fowl erythroblastosis virus [31]. Tumor-incidence results at "infinite" time also have been published for fowl lymphomatosis virus [32], polyoma virus in hamsters [33], the Bittner mouse mammary cancer virus [34], [35], and, in studies involving only 5 mice per dose group, the Friend mouse leukemia virus [36].

The only data of this group which fit a theoretical one-hit curve are those for the Friend mouse leukemia virus [36]. However, as already mentioned, only small numbers of animals were included in each dose group and the published results are therefore inadequate for judging the homogeneity of the test-animal population as a whole.

The results with rabbit papilloma virus [28] fit a one-hit curve over most of the significant dose range, but they depart from it at the higher doses (see [4] and [15]) in a manner similar to that illustrated in figures 6 and 11. The data of the remaining tumor viruses of this group show either relatively flat overall slopes, or abrupt changes in slope comparable to those illustrated for the Rous sarcoma virus. It appears therefore that in all of the animal-tumor virus systems thus far adequately investigated, heterogeneity in host susceptibility is a complicating factor in the study of dose-response relationships.

At the present time the only other tumor virus (besides the Rous sarcoma virus) which can be made to produce recognizable malignant changes in an in vitro system is the polyoma virus. Under the usual tissue culture conditions the polyoma virus produces only cell destruction [33], [37], [38], but special in vitro situations recently have been described [39], [40] which result in the development of cellular changes that are recognizable as malignant transformation. In vitro studies on carcinogenesis with this virus might therefore be expected in the future.

6. Virus replication

Another problem which it is essential to consider in the development of mathematical models of virus carcinogenesis is that of virus replication. The fact that viruses cannot be grown on artificial media, but are completely dependent upon living host cells for their replication, is the primary characteristic other than their much smaller size which separates viruses from other microbiological agents of disease. Viruses cannot be seen under an ordinary light microscope, but must be photographed with an electron beam in an electron microscope, and then further enlarged by photographic methods to a final magnification of about 30,000 to 50,000 fold in order to be resolved visually. The smallest viruses measure from about 10 to 15 mμ in diameter, whereas the largest ones measure about 300 mμ. In spite of their minute size, viruses have recognizable morphological characteristics and, like the much larger unicellular microorganisms, are composed of both genetic and nongenetic material. The genetic material of

viruses consists primarily of nucleic acid, which may be of either the ribose or deoxyribose type. The nucleic acid component is more electron-dense than the remainder of the virus particle and may be recognized in electron micrographs as a discrete inner structure designated as the nucleoid. The viral material surrounding the necleoid is composed largely of protein, though lipide may be contained in some of the larger viruses. It is less dense to electrons, and has been referred to by a variety of names, such as jacket, coat, sheath, shell, and soma. Many plant viruses are rod shaped, but some of them are spherical as are most of the animal viruses. Certain viruses which parasitize bacteria (bacteriophages) have a tadpole-like structure with a hexagonal "body" and a definite "tail," as represented diagrammatically in figure 17.

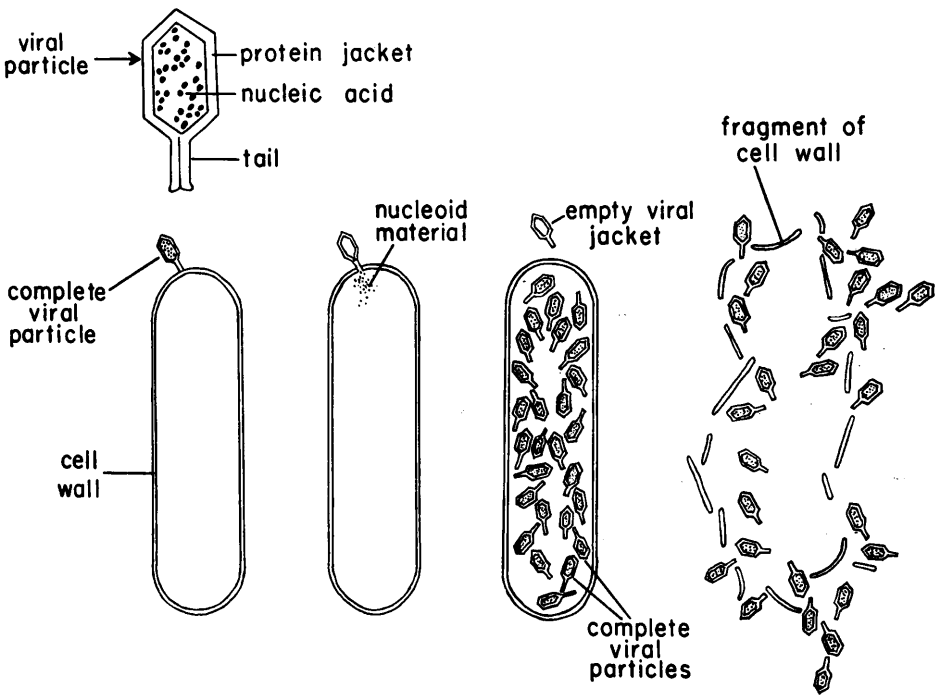

FIGURE 17

Diagrammatic representation of the replication of bacteriophage in a host bacterial cell.

Because their hosts are independent "free-living" unicellular organisms (bacteria), the bacteriophages have been more accessible to investigation than plant and animal viruses. Knowledge of their mechanism of replication is therefore considerably more advanced (see [5] and [41] to [44] for discussion and references). Figure 17 summarizes the mechanism diagrammatically for the T2 bacteriophage, which parasitizes ordinary colon bacilli. The phage particle does not enter the bacterial cell, but attaches itself to the cell wall by means of an

"enzymatic-adsorptive apparatus" [43] in a knob on the end of its tail. The genetic, or nucleoid material of the virus then passes through the hollow shaft of the tail into the bacterial protoplasm. After such "injection" of phage genetic material the empty soma plays no further role in phage replication, and it may actually be dislodged by vigorous stirring of the bacterial suspension without altering the subsequent course of events. The entire cycle of replication takes place within about 25–30 minutes at 37° C. Within 2 or 3 minutes after infection there is an alteration in the morphology of the bacterial nuclei and growth of *bacterial* cell material ceases. Certain metabolic processes continue, however, which result in the growth of both protein and nucleic acid "building blocks" of *virus* material, followed by the fabrication of complete phage particles. Finally, under the influence of the extraneous phage material, enzymes appear which bring about lysis of the cell walls of the host bacteria, and the cells "burst," thus liberating mature phage particles capable of infecting new host cells. The process of phage replication is thus one of fabrication by living host cells in accordance with specific patterns, or "information," supplied by the infecting phage genetic material.

The picture is not as complete for plant and animal viruses and it is not yet known whether entry of the viral soma into host cells takes place, or is necessary, for infection under natural conditions. However, it has been definitely established that the nucleic acid component, alone, of both plant [42], [45], [46] and animal [42], [47]–[49] viruses is capable of infecting appropriate host cells, and of causing them to manufacture complete virus of the same type and immunologic specificity as that from which the nucleic acid was derived.

Although information is lacking on the intracellular sites at which synthesis of viral protein and nucleic acid "building blocks" takes place, electron micrographic evidence suggests two different mechanisms by which fabrication of virus particles in their final or complete form is accomplished. The first mechanism, illustrated diagrammatically in figure 18, was revealed in studies on influenza virus (see [50] for references), but recently has been observed also in electron micrographic studies on neoplastic tissues resulting from infection with the Bittner mouse mammary cancer virus [51], [52], the fowl lymphomatosis virus [52], [53], the Friend mouse leukemia virus [54], and the Moloney mouse leukemia virus [55]. Aggregates of electron-dense material having the size and shape of the viral nucleoid appear just under the cell membrane, or just beneath the membrane of the endoplasmic reticulum, which is continuous with the cell membrane. This is followed by a protrusion of the membrane to form a microvillus containing the nucleoid, and finally by a constriction of the microvillus at its base, resulting in the budding off of the mature virus particle. The diagram of figure 18 is oversimplified in that an inner viral membrane, or even double membranes, may be present beneath the outer membrane of host substance [51], [54]. Also, recent studies indicate that a process of maturation of the nucleoid occurs just beneath the plasma membrane or of the endoplasmic reticulum membrane and to some extent after viral release [54], [55]. Such may be

seen with sufficiently high magnification and resolution in ultrathin-section electron micrographs. The outer shell of host material may be an important factor in accounting for the typical low antigenicity and high species specificity of these particular viruses.

A second mechanism of virus maturation appears to reside in the "condensation" of fully formed virus particles—except for extra membranes that may be

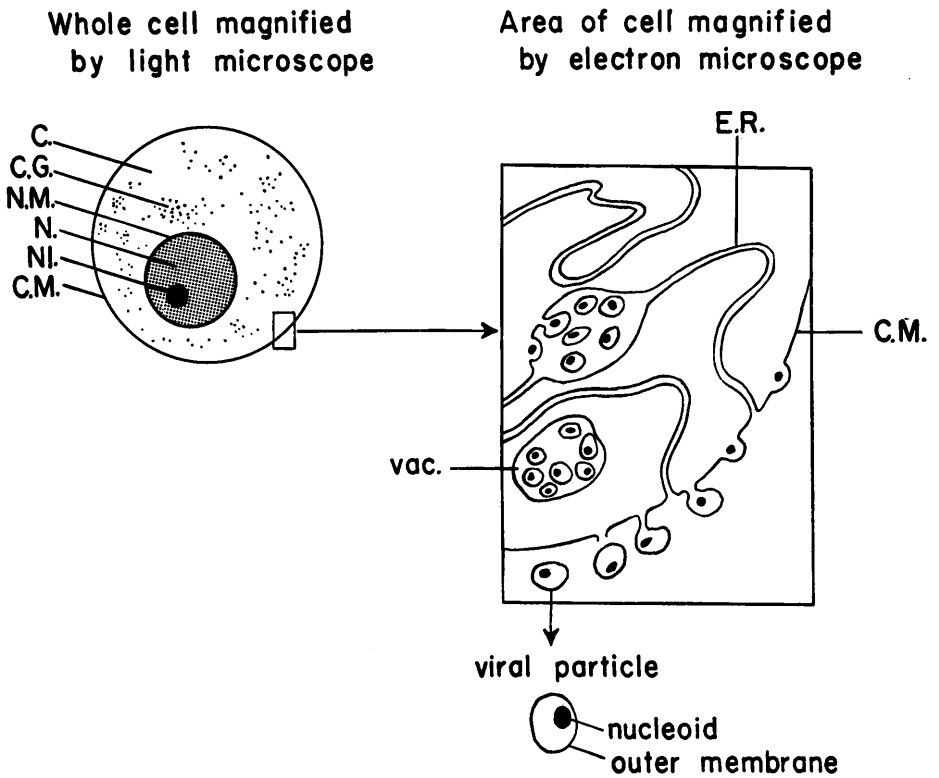

FIGURE 18

Diagrammatic representation of virus particle formation at cell membranes, characteristic of certain animal viruses. C, cytoplasm; C.G., cytoplasmic granules; N.M., nuclear membrane; N, nucleus; Nl, nucleolus; C.M., cytoplasmic membrane (cell membrane); vac., vacuole; E.R., endoplasmic reticulum.

picked up as the particles emerge through nuclear or cytoplasmic membranes—out of an electron-dense matrix material which previously accumulates in aggregates in the virus infected cells. Such aggregates may occur either in the nucleus [56]–[58] or the cytoplasm [44], [59]–[63], depending on the infecting virus. Also, they may, among different virus infections, be large enough to be readily recognized under a light microscope [44], [59], or they may be so small

as to require an electron micrograph for their distinction from normal, minute, cytoplasmic granules or organelles [60]–[63].

Figure 19 shows a diagrammatic representation of the "condensation" of mature virus particles as they appear to occur in fowl myeloblastosis [60], [61] and in the Rous sarcoma [62], [63]. The minute aggregates of amorphous matrix material are about the size of normal mitochondria and have been designated as "gray bodies" in electron micrographs [60]. The progression from "condensa-

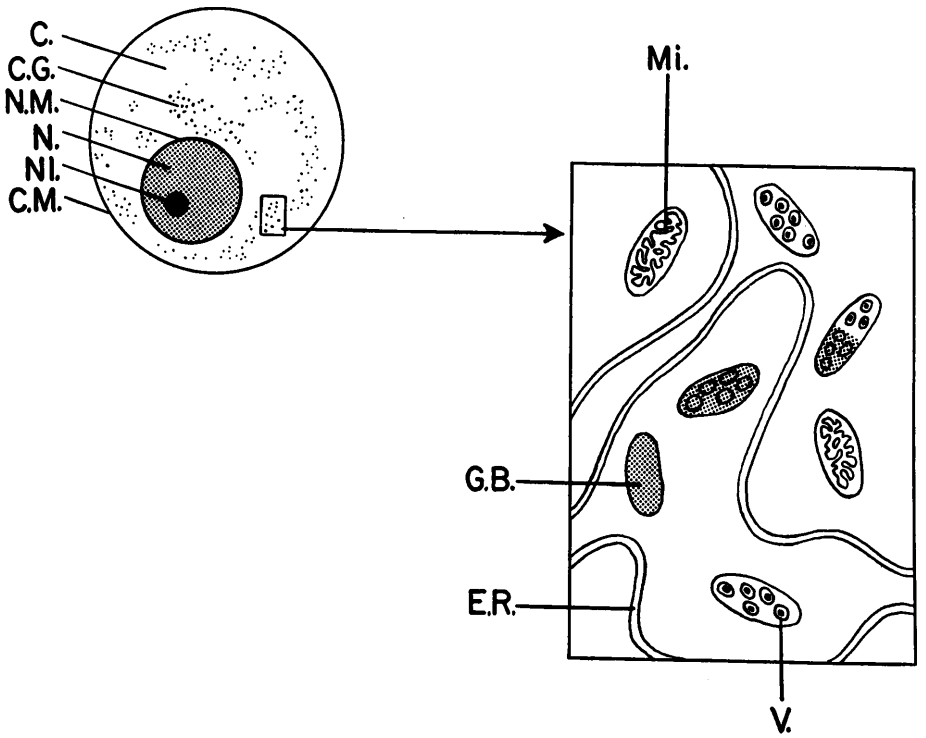

FIGURE 19

Diagrammatic representation of virus particle formation within matrix of amorphous precursor material, characteristic of certain animal viruses.
Mi, normal mitochondrium; G.B., "gray body" composed of amorphous matrix material; V., virus particle.

tion rings" in the amorphous matrix, to "vacuoles" containing mature particles but little or no matrix material is illustrated in figure 19.

Figure 20 illustrates the crystalline array of virus particles frequently observed with certain viruses that replicate within the nucleus, including the Shope papilloma [57] and polyoma [56] viruses.

The exact method of replication of viral subunits is not known at the present time, but it is widely believed that the replication is via a "template" copying

mechanism. The new subunits, particularly of viral nucleic acid, might act as additional "templates" thus accounting for the exponential growth observed with the rapidly acting viruses. However, replication of some of the subunits by growth and binary fission cannot be ruled out. It seems clear, though, from all evidence available at the present time, that the mature virus particles do not themselves undergo binary fission but break down into subunits prior to replication, and then are reassembled in increased numbers from replicated subunit material. As Luria [42] has summed up the problem, "virus multiplication as

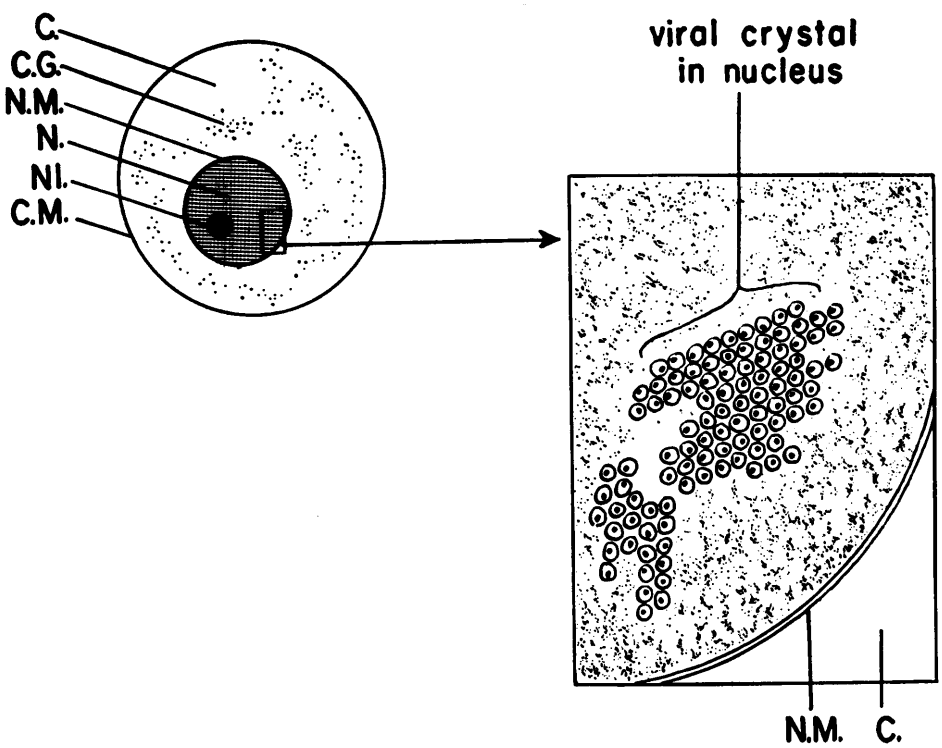

FIGURE 20

Diagrammatic representation of crystalline array of virus particles within the nucleus, characteristic for certain animal viruses.

a biological process belongs on the level of the replication of subcellular elements, that is, on the level of cell growth rather than of cell multiplication."

Although virus replication appears to occur exponentially at a rate independent of virus infecting dose in the case of bacteriophages and certain rapidly acting animal viruses, there is evidence with at least one tumor virus (virus of Rous sarcoma [64]) that the rate of virus multiplication in its animal host is dependent on the initiating dose of virus. The type of function which takes care of virus replication in mathematical models may not be the same for all viruses.

7. Concluding comments

It is reasonable to anticipate that, with appropriate models, mathematical projections of data acquired in studies on dose-response relationships and in kinetic studies on virus replication may some day allow accurate predictions to be made regarding the cancer-inducing potentialities of a given agent. This is of importance to public health research and to the human virus-cancer problem for two reasons. *First*, all known cancer viruses of animals are species specific and cannot be made to cross broad species barriers. Therefore, if viruses exist which cause cancer in humans they probably cannot be demonstrated by injections into laboratory animals, at least with knowledge available at the present time. Since human subjects cannot be used for biological tests no means now exist for the positive identifiction of human cancer viruses. *Second*, there is no dearth of viruses which could possibly be associated with human neoplasms. With the development of tissue culture techniques, a large number of new viruses has been isolated from human tissues during recent years, and additional ones are continuously being discovered. Many of them have not yet been identified with any known human disease [14]. The possibility exists that some of these "sleeping orphan" viruses may be responsible for the development of cancer after a characteristic "latent period." All approaches to the elucidation of the disease-producing potentialities of these agents, including the mathematical approach, should therefore be vigorously pursued.

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