ACTINOMYCIN D: EFFECTS

ON MOUSE L-CELLS

S. BACCHETTI *and* G. F. WHITMORE

From the Ontario Cancer Institute, Toronto 5, Ontario, Canada

ABSTRACT The lethal and inhibitory effects of actinomycin D (Act D) on asynchronous and synchronized populations of mouse L-cells have been studied. It has been shown that the survival curve of populations in the logarithmic phase of growth can be approximated by two exponential survival curves corresponding to a sensitive and resistant moiety. The size and sensitivity of both moieties vary during the growth of the population. As the cell population moves through logarithmic and into stationary phase, the sensitive moiety becomes smaller but more resistant whereas the resistant moiety increases in size and also becomes more resistant. This variation appears to be related to a reduced uptake of Act D and also a reduced rate of DNA and RNA synthesis. Variations in sensitivity to the drug have also been observed during the division cycle of synchronized cells with cells in the S phase showing the greatest uptake of the drug and also the greatest sensitivity. However, no direct correlation between uptake and sensitivity has been established. Actinomycin D has inhibitory effects on both RNA and DNA synthesis. RNA synthesis is inhibited rapidly but does not seem to drop to less than 5% of the control value. The inhibition of DNA synthesis appears to occur over a longer period and may reach values as low as 0.25% of control. In both cases the degree of inhibitions appears to be dependent on both the length of exposure and the concentration of the drug. Certain similarities between the response of cells to Act D and X-rays have been observed and are discussed.

INTRODUCTION

Much of the data which has been accumulated on the effects of actinomycin D (Act D)[1] consists of isolated observations of a single phenomenon in a variety of cellular and cell free systems and it is therefore sometimes difficult to correlate these into a meaningful picture of the action of the compound (for reviews see Reich and Goldberg, 1964; Goldberg, 1965). This seems especially true of attempts to correlate the effects of various concentrations of the drug on cell growth, cell survival, and macromolecular synthesis. Since the compound is often used as an inhibitor of RNA synthesis in attempts to determine the effect of such inhibition on other cell func-

---

[1] The following abbreviations are used in this article: Act D = actinomycin D; [3H]Act D = tritiated actinomycin D; CdR = deoxycytidine; TdR = thymidine; [3H]TdR = tritiated thymidine; [3H]UR = tritiated uridine; PBS = phosphate buffered saline; TCA = trichloroacetic acid.

tions, it seems particularly important to have a detailed knowledge of the effects of the compound on other biochemical processes and on cell survival. Also, because of the observations made by Elkind (1967; Elkind, Sakamoto and Kamper, 1968) that Act D may in some respects mimic the effects of radiation, it seemed that a study of the effects of the compound might prove useful to our understanding of the action of ionizing radiation. An additional reason for interest in this compound is its potential use as a cancer chemotherapeutic agent (Sugiura, 1960). Bruce (1967) has classified chemotherapeutic agents on the basis of their effects on rapidly proliferating mouse lymphoma cells and on normal mouse marrow cells. In this classification Act D is placed in Class III along with other agents whose toxicity for mammalian cells appears to be strongly dependent on whether the cells are actively progressing through the cell cycle. Radiation on the other hand appeared to fall into Class I amongst agents whose toxicity did not seem to be strongly related to the rate of progression through the cell cycle.

For the above reasons the studies outlined in this paper were undertaken using mouse L-cells as the test system. Our results indicate that Act D is a highly potent cytostatic and cytotoxic agent, that it does in some ways mimic the action of ionizing radiation, and that while it is a potent inhibitor of RNA synthesis, this inhibition only occurs at drug concentrations which have marked effects on DNA synthesis and cell viability.

MATERIALS AND METHODS

The experiments presented in this paper were carried out on a subline of Earle's L-cells, L60T (Till, Whitmore and Gulyas, 1963). For most experiments the cells were grown in suspension culture in medium 1066 Na$^-$Co$^-$ which consists of C.M.R.I. 1066 (Connaught Medical Research Laboratories, Toronto, Canada) (Parker, 1961) from which all of the coenzymes and nucleosides have been removed. This medium was supplemented with 10% (v/v) of calf serum (Flow Laboratories Inc., Rockville, Md.) which was dialysed three times against 20 volumes of PBS (Dulbecco, 1954). When synchronized populations were required, cells growing in medium 1066 lacking coenzymes and thymidine, but containing the other nucleosides (1066 T$^-$Co$^-$), were used; no difference in the cell doubling time or cell response to Act D has been observed in the two media or in media containing nondialysed fetal calf serum. The stock cultures were grown in glass spinner flasks (Johns Glass, Toronto, Canada) at 37°C and under these conditions the cell doubling time (T_D) in the logarithmic phase of growth was initially 16–18 hr, but during the course of this study it appeared to approach 14–16 hr as indicated in the Results.

Stock solutions of Act D (Merck, Sharp and Dohme, West Point, Pa.), 100 times concentrated, were freshly prepared before each experiment by dissolving the drug in medium with no serum under sterile conditions. No appreciable differences in activity were observed among different batches of the drug. For experimental purposes replicate 200 ml cultures were set up in spinner flasks at a concentration of about 5 × 10^4 cells/ml. Treatment of these cultures, unless otherwise indicated, was initiated when the cells were in the logarithmic phase of growth at a concentration between 1 and 2 × 10^5 cells/ml. In some experiments only a single determination of total cell number or viability at a fixed period of time following

treatment was required; in these cases replicate 10 ml cultures, obtained by dividing a single original culture, were set up in 17 × 100 mm plastic tubes (Falcon Plastics, Los Angeles, Calif.) and incubated in a rotating wheel at 37°C. The total cell number was determined by means of an electronic particle counter (Coulter Electronics, Chicago, Ill.). Viability was assayed by using the plating technique of Puck and Marcus (1955). After treatment with Act D the cell suspension, or aliquots of it, was washed three times by centrifugation and resuspension in equal volumes of fresh medium free of the drug; the final cell suspension was then counted and appropriately diluted and 1 ml aliquots were plated in 15 x 60 mm plastic Petri dishes (Falcon Plastics, Los Angeles) containing 5 ml of medium. In all experiments the plating medium consisted of C.M.R.L. 1066 supplemented with 10% (v/v) nondialysed fetal bovine serum (Flow Laboratories Inc., Rockville, Md.). The dishes were incubated at 37°C in an atmosphere containing 5% CO_2 ; after 10–12 days the resulting colonies were stained by addition of a 1% aqueous solution of methylene blue for 1 hr, rinsed and counted. Plating efficiency of untreated cells ranged from 70 to 100%.

In most of the experiments in which synchronized populations were used, synchrony of cell division was obtained with the [3]HTdR-suicide technique of Whitmore and Gulyas (1966). However, to study the uptake of [3]HAct D as a function of age in the cycle, the synchronized population was obtained by selection of mitotic cells (Robbins and Marcus, 1964).

Determination of the uptake of radioactive precursors for DNA and RNA synthesis in Act D-treated cells was carried out either with pulse labeling or continuous labeling techniques. In the first case, at various times after addition of the drug, 2 ml aliquots of cell suspension were incubated for 30 or 60 min at 37°C in the presence of either [3]HTdR (16.2 c/mmole or [3]HUR (15.3 c/mmole), both purchased from the Radiochemical Center, Amersham, England. When [3]HUR was used, 10 μg/ml of unlabeled TdR and CdR were also added to reduce labeling of the DNA. In the second type of experiment, the cells were continuously exposed to [3]HUR before and after addition of Act D; as a function of time, 2 ml aliquots were removed from the cultures for determination of the amount of radioactivity incorporated. In every case the uptake of label was terminated by the addition of 8 ml of cold (4°C) PBS containing 100 μg/ml of the unlabeled precursor. Duplicate samples of 4 ml each were filtered through Millipore filters, HAWP 02500, HA 0.45 μ (Millipore Filter Corp., Bedford, Mass.) which then were washed with cold PBS (twice), cold 5% TCA (twice), cold PBS (once), and cold 95% ethanol (twice). The Millipore membranes were allowed to dry and then counted in a liquid scintillation counter (Nuclear-Chicago Corporation, Des Plaines Ill.). In all experiments either [3]HUR or [3]HTdR was added to a sample of chilled cells and these samples were subjected to the same procedures outlined above. In every instance the amount of uptake was less than one per cent of the uptake in a control cell population. Also, in all cases tests indicated that isotope depletion during the course of the incubation was not a significant factor.

In some experiments, DNA and RNA syntheses were also measured by pulse labeling the cells for 1 hr with [32]P (36 μc/ml) (Atomic Energy of Canada, Chalk River, Ont., Canada). After incubation separation of the DNA and RNA fractions was performed according to the Schmidt-Tannhauser technique (Schmidt and Tannhauser, 1945) and aliquots were counted in the liquid scintillation counter.

The measurements of uptake of [3]HAct D (2.6–4.2 c/mmole) (Schwarz Bioresearch Inc., Orangeburg, N. Y.) were initially complicated by the tendency of the drug to bind to plastic and glass containers (Kessel and Wodinsky, 1968) and to be released in the presence of ethanol. In early experiments the following technique was used: 2 ml aliquots of cell suspension were incubated with [3]HAct D in a 5% CO_2 incubator at 37°C for the required time;

after addition of 8 ml of cold PBS the cells were centrifuged and resuspended twice in cold PBS and once in cold 95% ethanol; 0.1 or 0.5 ml aliquots of the ethanol suspension were then added to scintillation fluid and counted in the scintillation counter. However, control experiments, in which medium with no cells was incubated in the same way with ³HAct D, demonstrated that the drug bound to the container during the incubation period (apparently more efficiently to the plastic than to the glass containers) and was brought again into solution by the ethanol giving a high and somewhat variable background. The data obtained with this technique were corrected for this effect, but later experiments were performed with a filtration technique similar to the one used for ³HTdR or ³HUR incorporation. For ³HAct D, however, the filters presoaked in a solution of unlabeled Act D were washed twice with 10 ml of PBS and twice with 10 ml of ethanol, and not with TCA, since experiments in which ³HAct D was bound to purified DNA on Millipore filters indicated that TCA was able to break the binding between Act D and DNA. By doing all the washes on cells absorbed on Millipore filters, any ³HAct D bound to the container was not transferred to the Millipore filter and the background due to the medium was therefore much lower and constant.

X-irradiations were performed with a Picker-Vanguard X-ray machine (Picker X-Ray Corp., Cleveland, Ohio) operating at 280 kv and 20 ma at a half-value layer of 1.12 mm Cu, giving a dose rate of 1000 rad per min.

RESULTS

Inhibition of Growth by Act D

The effect of increasing concentrations of Act D on the growth of mouse L-cells is shown in Fig. 1. For concentrations up to 1×10^{-3} µg/ml, there appears to be a concentration-dependent slowing of the growth rate at early times, but after three or four generations the growth rate approaches that of the control population. This increase in growth rate at later times cannot be attributed to depletion of the Act D in the medium since the cells were diluted approximately every 50 hr into fresh medium containing the original concentration of Act D. At drug concentrations above 1×10^{-3} µg/ml, the cell number increases somewhat more slowly than the control for short periods of time, reaches a plateau, and then begins to fall off as the cells begin to lyse. If the concentration of Act D is increased to 0.5 or 1.0 µg/ml (not shown), cell growth continues at the control rate for 2–3 hr but is completely inhibited after 3–4 hr although no cell lysis is detectable for at least 12 hr. Similar results have been obtained with mouse fibroblasts L-929 (Reich et al., 1962) and with human cells growing in monolayers (Fraccaro et al., 1966; Kessel and Wodinsky, 1968).

Survival of Asynchronous Populations

If cells are exposed to various concentrations of the drug for periods of 1 hr and their proliferative capacity determined by plating, results similar to those shown in Fig. 2 are obtained. Such survival curves are almost invariably biphasic and for the example shown, which was obtained for cells in logarithmic growth, the C_{10} (the concentration required to reduce survival to 10% during a 1 hr exposure) is 0.23 µg/ml for the initial slope and 1.7 µg/ml for the more resistant fraction.
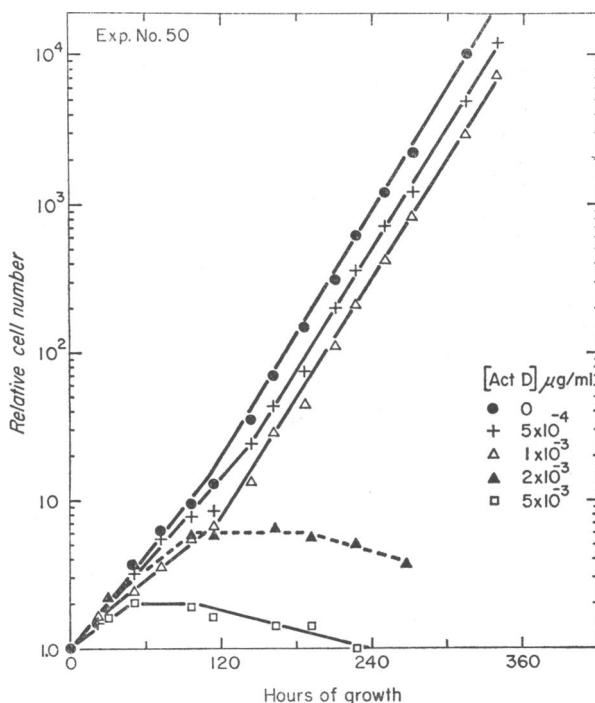
FIGURE 1 The effect of various concentrations of Act D on the growth of L-cells. Act D was added at time zero to a series of replicate cultures in early logarithmic phase of growth and the increase in cell number was recorded thereafter by means of an electronic counter.

If, for a range of concentrations of Act D, the survival of cells is plotted as a function of the duration of the exposure, biphasic curves are again obtained (Fig. 3). For drug concentrations above 0.1 μg/ml it is apparent that the drug begins to exert its toxic effect almost immediately and other experiments have indicated that exposures of 5–10 min will kill 20–40% of the initial population. For drug concentrations of 0.05 μg/ml and less, the curves of survival versus time show very little decline at early times, followed by a period of more rapid decline beginning after 4–8 hr of exposure to the drug.

The survival curves illustrated in Figs. 2 and 3 were all obtained using cells in the logarithmic phase of growth, as determined by doubling time measurements. However, in repeating some of these experiments it was observed that the survival curves were somewhat variable and that the sensitivities and perhaps the relative proportions of the sensitive and resistant moieties varied. This suggested that the response of the populations was in some way related to their over-all metabolic rate, and perhaps to the age of the population with respect to lag, logarithmic, and stationary phases. In an attempt to determine to what extent this hypothesis was correct, an experiment was carried out in which a population in stationary phase was diluted approximately 8-fold into fresh growth medium and then, as a function of time, a
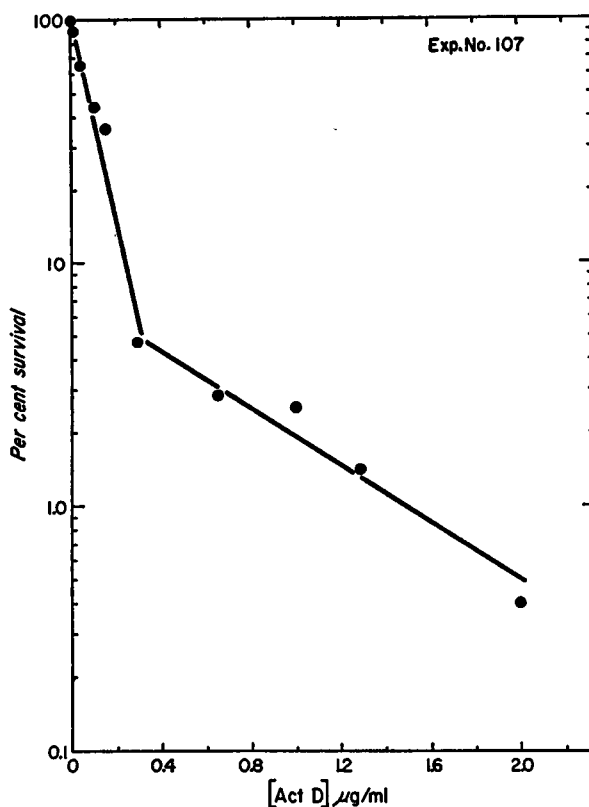
FIGURE 2 The effect of various concentrations of Act D on the viability of mouse L-cells during a 1 hr incubation in the presence of the drug. Replicate cultures in the logarithmic phase of growth were incubated for 1 hr with Act D, washed, diluted, and plated for survival; a control culture, with no drug, received the same treatment. The per cent survival, relative to this control culture, is plotted versus increasing concentrations of Act D.

number of determinations were made on the cell population. These determinations included: survival after a 1 hr exposure to Act D at concentrations of 0.5, 1, 2, and 4 μg/ml; rate of DNA and RNA synthesis as determined by the uptake of ^{32}P and ^{3}HUR during a 1 hr pulse; and the uptake of ^{3}HAct D during a 1 hr pulse. In panel a of Fig. 4, the growth curve of the population during the course of the experiment is illustrated, with the arrow at time zero indicating the cell count before the dilution. Fig. 5 shows the cell survival data plotted as a function of Act D concentration at different times after dilution. It is apparent that after dilution the cells become progresssively more sensitive to the action of the compound, until they reach a maximum sensitivity between 20 and 40 hr after dilution. At later times both the sensitive and resistant fractions become progressively more resistant to the action of the drug, and there is some indication that the proportion of resistant cells is increasing. One conclusion which seems to be evident from these survival studies is
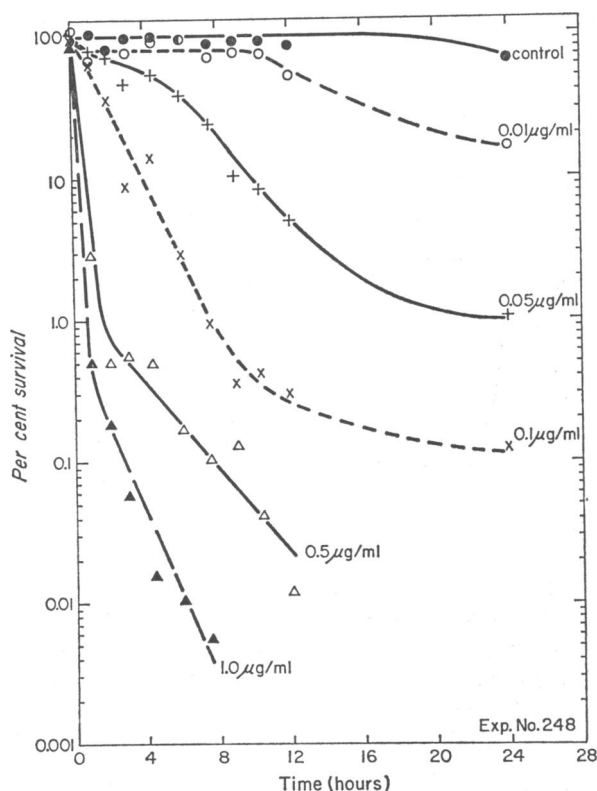
FIGURE 3 The effect of various concentrations of Act D on the viability of mouse L-cells as a function of the duration of exposure to the drug. Replicate cultures in logarithmic phase of growth were treated with the drug at time zero and at intervals thereafter aliquots from each culture and from an untreated control were washed, diluted, and plated for survival. The per cent survival relative to the survival of the control at time zero is plotted versus the duration of exposure to the drug for each concentration.

that the sensitivity to Act D is a strong function of the age of the population and even appears to show pronounced variation throughout what, on the basis of cell number determinations, would normally be called the logarithmic phase. It should also be pointed out that the variation in sensitivity is not a result of depletion of Act D by the increased cell population, since control experiments have indicated that this is not a factor. The most likely explanation of these results would appear to be that there have been changes in the proportion of cells in various parts of the cell cycle which might bring about changes in the rates of DNA and RNA synthesis, uptake of Act D, etc.

In an attempt to relate the changes in sensitivity to rate of DNA or RNA synthesis, or to the rate of uptake of Act D, these rates were also measured as a function of time in the same experiment. Panel *b* in Fig. 4 illustrates the amount of uptake of ³HAct D during a 1 hr exposure as measured by the filtration technique. It can be
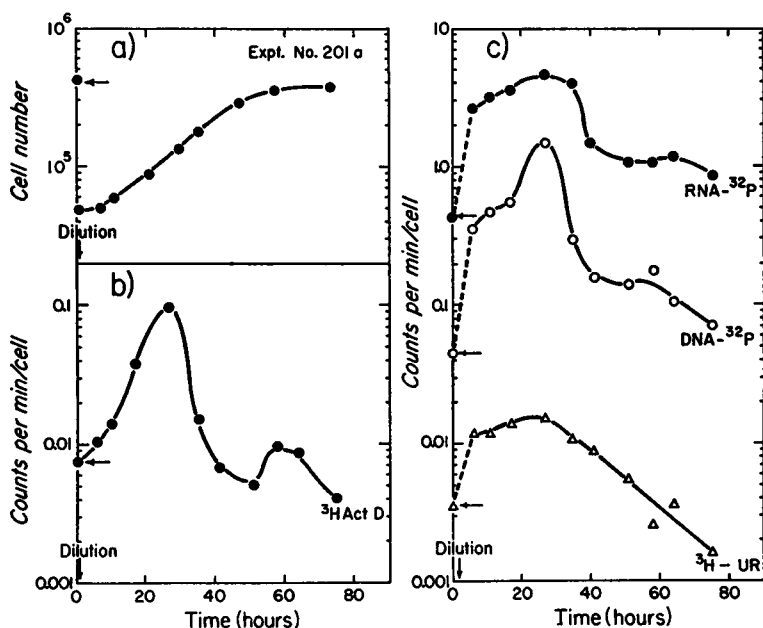
FIGURE 4 Panel a: Cell number per ml of an untreated population of mouse L-cells after dilution from stationary phase. Panel b: incorporation of ³HAct D (2 μc/ml, 0.8 μg/ml) during a 1 hr pulse in aliquots of the above population during its growth. Panel c: uptake of ³²P (36 μc/ml) in the RNA and DNA fractions and of ³HUR (15.3 c/mmole; 16 μc/ml) in the acid precipitable material in aliquots of the population of panel a during a 1 hr pulse at different times of growth. In each panel the measurements before dilution of the population are indicated by arrows on the ordinate axis.

seen that the rate of uptake is a minimum immediately after dilution, but that it increases rapidly and reaches a maximum at about 25 hr, or about the same time as the cells exhibit their maximum sensitivity to the drug. Panel c of Fig. 4 shows the uptake of ³²P into DNA and RNA during a 1 hr pulse as a function of time, and also shows the uptake of ³HUR during a 1 hr pulse; none of the populations were exposed to Act D. All of these uptake data show that the rates of synthesis are low immediately after dilution, but then increase, reaching maximum values about the middle of the logarithmic phase, and finally begin to decrease. The fact that the times of maximum uptake of Act D and incorporation of labels into DNA and RNA all approximately correspond, makes it difficult to attempt to correlate sensitivity to Act D to any one of these parameters, although the data of Fig. 5, if plotted as per cent survival versus time after dilution, show a strong inverse correlation between survival and uptake of Act D. It should also be pointed out here that the initial points in panel c, those indicated by arrows, are uptake measurements made immediately prior to dilution, whereas the second point in each of the uptake curves was taken about 6 hr after dilution. Superficially these data would suggest that there is a sudden increase in the rate of synthesis of DNA and RNA, but more careful examination in
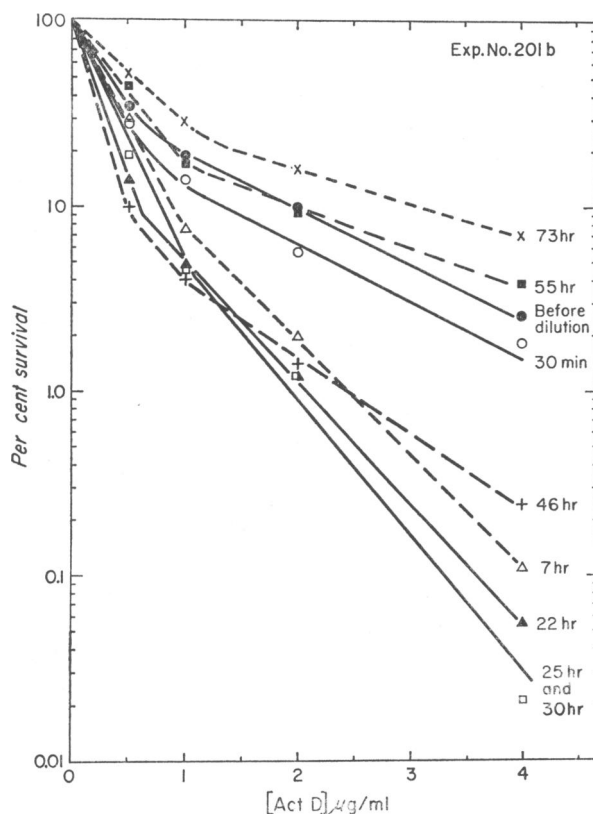
1434

FIGURE 5 Survival of cells exposed for 1 hr to different concentrations of Act D at various times after dilution.

control cultures of samples taken within 15 min after dilution makes it almost certain that part of this sudden increase is due to change in the size of various precursor pools; therefore, the change in rates of macromolecular synthesis may not be quite as extensive as this data would tend to indicate. Variations in pool sizes might also account for the differences observed between the uptake of ^{3}HUR and ^{32}P into RNA. During the course of the experiments outlined in this paper, no successful method of overcoming this pool problem or of exactly determining the magnitude of the effect was discovered. Some further evidence, however, that there are real changes in the rates of macromolecular synthesis comes from the work of Kim,[2] who has shown changes in the level of thymidine kinase in mouse L-cells during their growth which would roughly parallel the uptake of ^{32}P into DNA shown in the present experiments.

Following the above observation of the pronounced changes which occur in our cell populations during logarithmic phase, more strenuous efforts were made to keep

---

[2] S. C. Kim. 1967. Personal communication.

the cells in a more stable condition. This was accomplished by diluting the populations approximately every 20 hr and where possible, using these cells for experiments approximately 20 hr after dilution. One possible consequence of this procedure was that the doubling time of the population appeared to decrease progressively throughout these experiments from an initial value of between 16 and 18 hr to a final value of between 14 and 16 hr.

Effects on RNA and DNA Synthesis

The Act D-induced inhibition of the uptake of ^{3}HUR into RNA is shown in Fig. 6. In this experiment the drug was added at various concentrations at time zero to replicate logarithmic phase cultures and, at intervals thereafter, samples from each culture were incubated for 30 min in the presence of the labeled precursor. At all concentrations of the drug above 0.01 μg/ml, the uptake of the precursor is appreciably reduced by the action of the drug.

In experiments in which exponentially growing cells were prelabeled for 1 hr with ^{3}HUR and then Act D (0.05 or 0.1 μg/ml) was added to the cells still in the presence of the label, uptake appeared to continue for approximately 20 min at the control
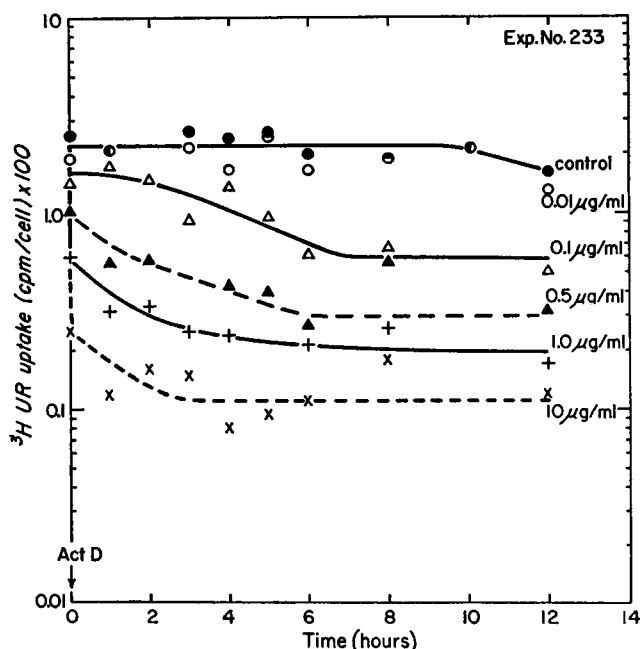

FIGURE 6 The uptake of ^{3}HUR (15.3 c/mmole, 6 μc/ml) during a 30 min pulse in logarithmic phase cultures to which Act D was added at time zero. The uptake in the acid precipitable material is plotted as counts per minute (cpm) per cell versus length of exposure to the drug. The points are shown at the beginning of the pulse.
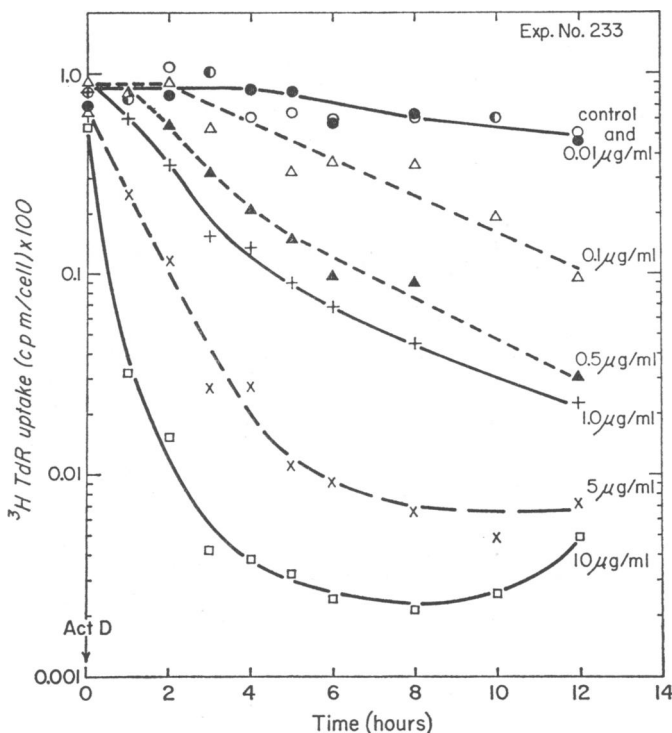
FIGURE 7 The effect of various concentrations of Act D on the uptake of ³HTdR (16.2 c/mmole, 6 μc/ml) in the acid precipitable material of logarithmically growing cultures. Act D was added at time zero and the uptake of the isotope was measured at intervals thereafter during 30 min pulses. The points are shown at the beginning of the pulse.

rate and then showed a marked decrease. With concentrations of 1 μg/ml and higher, uptake ceased immediately upon addition of the drug and then there appeared to be a loss of previously incorporated material. Results similar to these have already been reported for other mammalian cell lines (Scherrer, Latham, and Darnell, 1963; Prevec, 1965; Mueller and Kajiwara, 1966; Tobey et al., 1966).

The effect of Act D on the uptake of ³HTdR into DNA is illustrated in Fig. 7. The experimental design here is essentially similar to that used to obtain the data in Fig. 6 and a comparison of the two indicates that the inhibition of DNA synthesis takes longer to establish, but that once it is completely established it is more effective than the inhibition of RNA synthesis. Therefore, in the range of concentrations used in our experiments, Act D is a very efficient inhibitor of DNA as well as RNA synthesis.

Act D Sensitivity as a Function of Age in the Cell Cycle

Because of the pronounced variation in sensitivity to Act D exhibited by cells during logarithmic and stationary phase, and because of the suggestion by Elkind (1967)
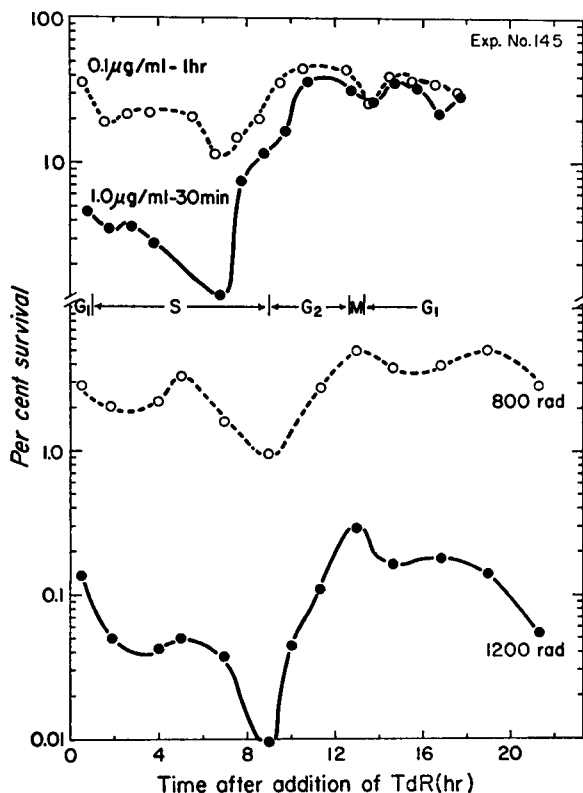
FIGURE 8 Age-dependent sensitivity of mouse L-cells to Act D and X-rays. Synchroniza-
tion of the cell population was obtained with ³HTdR treatment and reversal by addition of
TdR. At intervals after reversal, aliquots of the culture were treated with Act D (0.1 μg/ml
for 1 hr or 1 μg/ml for 30 min). The data for radiation survival are taken from Whitmore et al.,
1965. The relative position of the cells in the cycle at the time of treatment is indicated.

that Act D and radiation appeared to cause similar types of damage in mammalian
cells, it became of interest to determine sensitivity to the drug as a function of cell
age in the division cycle. For this purpose synchronized populations were obtained
by the use of the ³HTdR-suicide technique (Whitmore and Gulyas, 1966). This in-
volved exposing logarithmic phase populations growing in 1066 T⁻Co⁻ to ³HTdR at
a specific activity of 16.2c/mmole and a concentration of 2 μc/ml for 6 hr and then
stopping uptake of the label by the addition of unlabeled thymidine at a concen-
tration of 100 μg/ml. When this technique is used, all cells which pass through the S
phase are killed by the incorporated radioactive thymidine and therefore, after the
6 hr exposure to the radioactivity, the only viable cells in the population are con-
tained in a narrow window, 2–4 hr wide, at the end of the G_1 phase. After the
synchronization procedure cells can be exposed to the action of Act D at any time.
in the cell cycle.

The top part of Fig. 8 illustrates the results of such an experiment in which aliquots from a synchronized population with a doubling time of 16–18 hr were exposed to Act D, either 0.1 μg/ml for 1 hr or 1.0 μg/ml for 30 min at various times in the cell cycle. It is evident that the survival varies as a function of cell age and that as cells move from G_1 into S phase, they become more sensitive and in this experiment they appeared to gain their maximum sensitivity in middle to late S phase. The exact time during S phase at which the cells show their maximum sensitivity appeared to vary in different experiments over a period of approximately 3 hr around the middle of S phase. After this period of maximum sensitivity the cells rapidly became resistant until in G_2 they appeared very insensitive to the drug. The fact that in late G_2, M, and early G_1 the two vastly different concentrations of the drug, present for times which differed only by a factor of two, produced almost identical survival levels, may perhaps indicate that there is a small fraction of the cell cycle during which the cells are almost totally resistant to the action of the drug.

For purposes of comparison the bottom part of Fig. 8 shows the survival curves as a function of cell age for L-cells exposed to either 800 or 1200 rad of X-rays (Whitmore, Gulyas and Botond, 1965). Although the radiation and drug experiments were not carried out at the same time, the same synchronization procedure was used in both cases, and it is apparent that even though the survival levels for the two agents are rather different, there are some similarities in the response to the two agents as a function of cell age. In both cases the maximum sensitivity appears to occur in the S phase, although earlier for Act D, and in both cases G_1 and G_2 cells appear relatively insensitive.

Uptake of ^3H Act D

Preliminary attempts have been made to measure the uptake of Act D into L-cells both in asynchronous and synchronized populations. It must be born in mind in all of these studies that uptake has an operational definition, in that it is the amount of Act D which remains in the cell after the various washing procedures used to remove free labeled material from the medium. What is not clear is to what extent these measurements are an accurate reflection of the binding to critical sites within the cell. None of the washing procedures involved the use of TCA, since this compound breaks the binding between Act D and DNA (see Materials and Methods).

Figure 9 shows the uptake of Act D into an asynchronous population taken 25 hr after the previous dilution and incubated for various lengths of time with a total concentration of 1 μg/ml of Act D containing 4 μc/ml of ^3H Act D. It is apparent that over a period of approximately 60 min, the binding of the compound increases, but that the rate of incorporation is decreasing with time. This decrease with time is not due to depletion of the radioactive material, since appropriate controls have shown that there is no appreciable depletion of the total available Act D in the medium during the course of such an experiment. Similar results have been observed
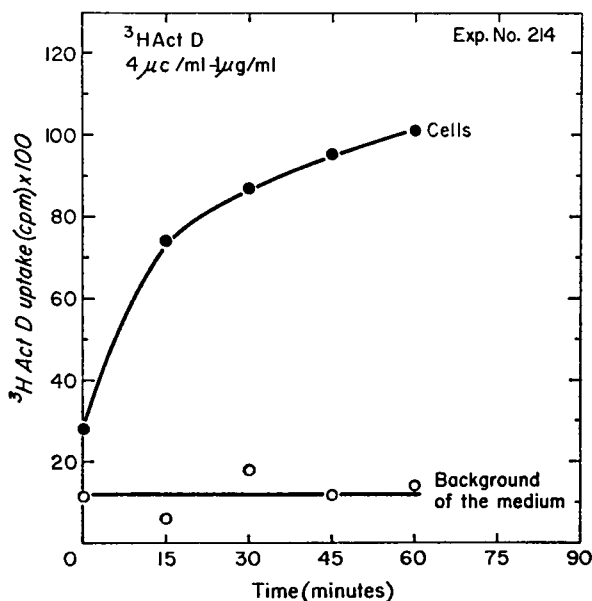
FIGURE 9 Uptake of ³HAct D in an asynchronous population of mouse L-cells versus time of exposure to the drug. Cells were exposed from time zero to ³HAct D (4 μc/ml, 1 μg/ml) and at various times aliquots from the culture were diluted with cold PBS and filtered on Millipore filters presoaked in a solution of unlabeled Act D. The filters were then washed with PBS and ethanol. The curve labeled "background of the medium" refers to the radioactivity measured in a similar way in a medium containing ³HAct D but no cells.

with other mouse and human cell lines (Fraccaro et al., 1966; Kessel and Wodinsky, 1968), but in the case of Chinese hamster cells the rate of uptake remained linear over a period of 2–3 hr (Elkind et al., 1968). In experiments in which we have determined the uptake as a function of concentration during a 1 hr exposure to the drug, it was also found that for concentrations of the drug above approximately 2 μg/ml, the rate of uptake was less than linear with concentration. This observation is again in contrast to results obtained with Chinese hamster cells in which it has been reported that the rate of incorporation over a 1 hr exposure is linear up to concentrations of 6 μg/ml (Elkind et al., 1968). The results of both of the above experiments with L-cells would suggest the existence of some sort of saturation phenomenon.

The age-dependent uptake of ³HAct D has also been studied in cell populations synchronized by the mitotic detachment technique (Robbins and Marcus, 1964); the results are shown in Figure 10, together with data on the uptake of ³HTdR into other aliquots of the same population. Although it is true that cells in the S phase which are most sensitive to the lethal effects of Act D also take up the most label, there is also considerable uptake in G_2 when the cells are relatively resistant. It is possible, however, that part of the uptake which we are measuring is not uptake into critical structures within the cell, and it is also possible that some of the Act D which
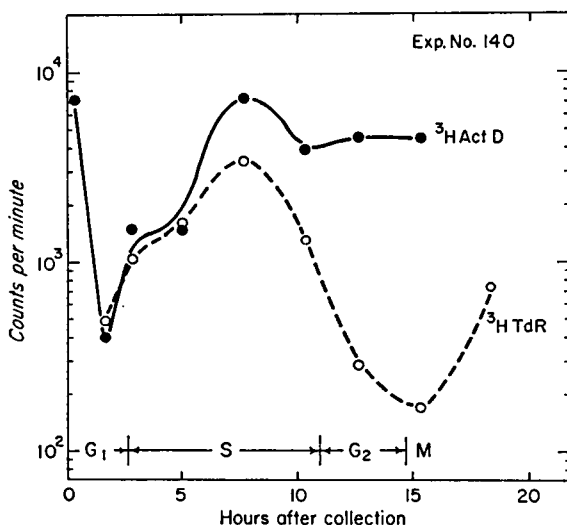
FIGURE 10. The uptake of ³HAct D (4 μc/ml; 1 μg/ml) in mouse L-cells during a 30 min pulse as a function of their age in the cell cycle. Synchrony was obtained by mitotic collection. The radioactivity present in the cell was measured by counting aliquots prepared by the centrifugation method described in the text. The uptake of ³HTdR into the acid precipitable material during a 30 min pulse is also given for comparison.

is taken up in various phases of the cell cycle is lost when the cells are diluted in plating experiments.

DISCUSSION

The effects of Act D on mouse L-cells growing in suspension culture are similar to the effects reported for other cell lines (Fraccaro et al., 1966; Elkind et al., 1968). At concentrations between 1 and 5 × 10⁻³ μg/ml, the drug markedly reduces the growth rate of L-cells after several generations but has essentially no effect on cell viability during exposures of 24 hr or less. At higher concentrations cell division is inhibited after 3–4 hr and cell lysis can be observed at later times. This observation suggests that cells in the G_2 phase are relatively resistant to the toxic effects of the compound and this conclusion has been born out by the studies on the age response of L-cells to the drug, which indicate that over the concentration range tested, cells in the S phase are the most sensitive (Fig. 8).

The studies reported here give ample evidence that the toxic response of L-cells to Act D is strongly dependent upon the age of the cell in the cell cycle and also on some other factor which appears to be correlated with the over-all rate of proliferation of the population. This can be most easily seen in the data of Fig. 5. For cells in the logarithmic phase the sensitivity to the drug is the greatest, but even in these populations it is apparent that not all cells have the same sensitivity and the survival curve can be approximated by two exponential survival curves. As cells progress

from logarithmic toward stationary phase the survival curves, as a function of Act D concentration, undergo several changes. A simple breakdown of each survival curve into two exponential survival curves indicates that the C_{10} of the sensitive fraction varies by a factor of approximately two throughout the logarithmic and stationary phases whereas the C_{10} of the resistant fraction varies by a factor of almost four. During this same time interval the size of the resistant fraction varies from approximately 10% of the population to a maximum of 40% of the population. Our attempts to relate these changes to changes in the over-all rate of DNA and RNA synthesis in the culture have not successfully demonstrated a correlation with the rate of synthesis of either compound. However, it does appear that at the time when cells are rapidly synthesizing DNA and RNA, they are also most sensitive to Act D.

Several mechanisms or combinations of mechanisms could be advanced to explain the resistance of G_2 and stationary phase cells to the toxic effects of Act D. It has been well established that Act D binds to DNA and it is this binding which is apparently responsible for the rapid inhibition of RNA synthesis (Reich and Goldberg, 1964; Goldberg, 1965). It seems reasonable to assume that the lethal effects of the drug are at least partially determined by the amount of this binding. If this is the case, then anything which alters the capability of the DNA to bind Act D or which alters the ability of Act D to reach the DNA might be expected to change the sensitivity of cells to the drug. Our experiments on the toxic effect of the compound and the ability of cells to take up labeled Act D (Figs. 9 and 10) would suggest that cells which take up the most Act D will in general show the greatest toxic effect. However, there are situations, notably for cells in the G_2 phase, when there appears to be appreciable uptake of the drug and yet the cells appear relatively resistant. One could explain this observation as well as all of the other variations of sensitivity by assuming that there are phases in the cell cycle when the binding of Act D has little effect on the cell because the drug is bound to sites which are not actively being utilized at the time, and before these sites are actively utilized there are opportunities for some of the Act D to be released. To explain the gradual shift in the proportion of resistant cells in the population, we have assumed that as cells progress into stationary phase, there is a redistribution of cells amongst the various phases of the division cycle. The observations discussed here are also in agreement with the work of Bruce (1967), who has suggested that the toxic effects of Act D are dependent upon the rate of cell proliferation.

In addition to studies on the toxicity of Act D, we have also studied the effect of the compound on the synthesis of DNA and RNA. It is well established that Act D binds to specific sites in DNA and that in some way this prevents the use of the DNA as a template for RNA polymerase (Reich and Goldberg, 1964; Goldberg, 1965). It has also been reported that Act D in higher concentrations is able to inhibit DNA polymerase (Reich and Goldberg, 1964; Goldberg, 1965). The range of concentrations of Act D used in our experiments appears to be inhibitory for both RNA and DNA synthesis, although the magnitude and time courses of the two inhibitions are

quite different. We were, however, never able to find a concentration of Act D which caused a significant inhibition of RNA synthesis without affecting DNA synthesis and cell viability as well. Similar observations have also been reported for HeLa cells (Kim, Perez and Djordjevic, 1968). In Chinese hamster cells, however, there appears to be a concentration range which inhibits RNA synthesis with the absence of significant effects on DNA synthesis or cell viability.[3]

Because of the effect on both RNA and DNA synthesis, it is impossible to determine which biochemical process, if either, is responsible for the toxicity of the compound. The prompt effect of the compound on both survival and RNA synthesis might be an indication that the cells are killed due to an interference with RNA synthesis; however, since some Act D may remain bound to DNA for long periods of time, other mechanisms of cell killing are probably equally likely.

Elkind (Elkind, 1967; Elkind et al., 1968) has reported that there appears to be a close similarity between many of the effects observed with radiation and with Act D. The experiments reported here appear to support this contention. Both agents appear to be more toxic for cells which are in the logarithmic phase than for cells in the stationary phase (Whitmore and Gulyas, 1967). In both Chinese hamster cells (Elkind et al., 1968) and L-cells the two agents show similar patterns of response around the cell cycle even though the patterns vary between the two cell lines. It must, however, be stressed that while there are similarities between the two agents there are also differences. Act D shows a greater variation in its toxicity than do X-rays as the cells pass through the logarithmic and into the stationary phase. The sensitivity to Act D appears to reach its maximum earlier in the cell cycle than the sensitivity to X-rays does. These differences do not rule out the hypothesis that the two agents have similar modes of action. With either agent it seems reasonable to assume that the overall damage observed will be a function of how much damage is done to the primary target, how much use is being made of this target by the cell at the time the damage is inflicted, and how much repair or relaxation of damage can occur before the sensitive target is used by the cell.

If this assumption is accepted, then even though the two agents have similar targets or sites of action, variations in the first or last of the three determining factors mentioned above could bring about variations in the response to the agents. For example it may be that DNA is the sensitive target for both agents but that the susceptability of DNA to attack by Act D is very dependent upon the condition of the DNA during the time of exposure to the drug; it may also be that the permeability of the cell to the drug varies throughout the cell cycle or it may be that even though Act D is bound to DNA this binding is sufficiently weak that some of the drug is lost before the DNA is used either for DNA or RNA replication. This latter assumption could also account for the observation that the toxic effect of Act D is not closely correlated with the amount of the drug taken up by the cells.

---

[3] W. K. Sinclair. 1968. Personal communication.

It might be assumed that the similarity of action between Act D and radiation might prove useful in attempts to determine the nature of the radiation-induced cell lesions responsible for cell killing, especially since a great deal is known about the binding sites of Act D within the cell. However, both agents appear to disrupt a number of processes within the cell and it may be difficult to decide which of these processes is the key to the toxic effects. Alterations in cellular properties which are not obvious to the investigator may be of extreme importance to cell survival whereas the converse need not necessarily be true.

Actinomycin is often assumed to be a specific inhibitor of RNA synthesis (Reich and Goldberg, 1964; Goldberg, 1965) and used to test for the effect of RNA inhibition in a variety of experimental situations. The data presented here would suggest that, in the absence of the determination of its effect on DNA synthesis and cell viability, this may be doubtful assumption.

REFERENCES

BRUCE, W. R. 1967. *In* Canadian Cancer Conference. Pergamon of Canada, Ltd., Toronto. 7:53.

DULBECCO, R., and M. VOGT. 1954. *J. Exp. Med.* 99:167.

ELKIND, M. M. 1967. *In* Radiation Research. G. Silini, editor. North Holland Publishing Company, Amsterdam. 558.

ELKIND, M. M., K. SAKAMOTO, and C. KAMPER. 1968. *Cell and Tissue Kinet.* 1:209.

FRACCARO, M., A. MANNINI, L. TIEPOLO, and A. ALBERTINI. 1966. *Exp. Cell Res.* 43:136.

GOLDBERG, I. H. 1965. *Amer. J. Med.* 39:722.

KESSEL, D., and I. WODINSKY. 1968. *Biochem. Pharmacol.* 17:161.

KIM, J. H., A. G. PEREZ, and B. DJORDJEVIC. 1968. *Cancer Res.* 28:2443.

MUELLER, G. C., and K. KAJIWARA. 1966. *Biochim. Biophys. Acta.* 119:557.

PARKER, R. C. 1961. Methods of Tissue Cultures. Hoeber-Harper, New York. 3rd Edition.

PREVEC, L. 1965. RNA Synthesis in L-cells partially synchronized with FUdR. Ph.D. Thesis. University of Toronto, Toronto.

PUCK, T. T., and P. I. MARCUS. 1955. *Proc. Nat. Acad. Sci. U.S.A.* 41:432.

REICH, E., R. M. FRANKLIN, A. J. SHATKIN, and E. L. TATUM. 1962. *Proc. Nat. Acad. Sci. U.S.A.* 48:1238.

REICH, E., and I. H. GOLDBERG. 1964. *In* Progress in Nucleic Acid Research and Molecular Biology. Academic Press, Inc., New York. 3:183.

ROBBINS, E., and P. I. MARCUS. 1964. *Science (Washington).* 144:1152.

SCHERRER, K., H. LATHAM, and J. E. DARNELL. 1963. *Proc. Nat. Acad. Sci. U.S.A.* 49:240.

SCHMIDT, G., and S. J. TANNHAUSER. 1945. *J. Biol. Chem.* 161:83.

SUGIURA, K. 1960. *Ann. N.Y. Acad. Sci.* **89**:368.

TILL, J. E., G. F. WHITMORE, and S. GULYAS. 1963. *Biochim. Biophys. Acta* **72**:277.

TOBEY, R. A., D. F. PETERSEN, E. C. ANDERSON, and T. T. PUCK. 1966. *Biophys. J.* **6**:567.

WHITMORE, G. F., S. GULYAS, and J. BOTOND. 1965. *In* Cellular Radiation Biology. The Williams & Wilkins Company, Baltimore. 423.

WHITMORE, G. F., and S. GULYAS. 1966. *Science (Washington).* **151**:691.

WHITMORE, G. F., and S. GULYAS. 1967. *In* Canadian Cancer Conference. 370, Pergamon of Canada, Ltd., Toronto. **7**:370.