A COMPARISON OF THE KILLING OF CULTURED MAMMALIAN CELLS INDUCED BY DECAY OF INCORPORATED TRITIATED MOLECULES AT −196°C

H. JOHN BURKI *and* S. OKADA

From the Division of Experimental Radiology of the Department of Radiology and the Department of Radiation Biology and Biophysics of The University of Rochester Medical Center, Rochester, New York 14620. Dr. Burki's present address is the Donner Laboratory, University of California, Berkeley, California 94720.

ABSTRACT The killing efficiency of tritium disintegrations in frozen mammalian cells labeled with tritiated uridine, histidine, and lysine was compared with the killing efficiency of incorporated tritiated thymidine. In each case, the distribution of tritium in the cells was determined by chemical fractionation as well as by radioautography. Of all tritium disintegrations, by far the most effective were those occurring in DNA molecules within frozen cells; such incorporated tritium has a killing efficiency of 0.006. When cells were incubated with tritiated uridine for 10 min to label nuclear RNA, the killing efficiency was 0.0015. When the cells were pulse labeled with tritiated uridine and permitted to grow in nonradioactive media for 10 hr before freezing in order to incorporate tritium into cytoplasmic RNA, the killing efficiency was reduced to 0.0010. The results suggest that decay of tritium in nuclear RNA is more effective than that in cytoplasmic RNA. When the cells were labeled with tritiated histidine or lysine for 30 min, tritium atoms were found mainly in the acid soluble rather than in the protein fraction and the killing efficiency in each case was approximately 0.0007. The results of these suicide experiments indicate that the killing efficiency of tritium disintegrations depends on where tritium is located within the cells. Tritium disintegrations in the nucleus are more effective in killing the cell than that in cytoplasm; and tritium disintegrations on DNA in the nucleus is more effective in killing the cell than that of nuclear RNA.

INTRODUCTION

Since many different tritiated compounds are now available, one can choose appropriate ^{3}H-labeled compounds to label specific types of intracellular macromolecules. "Suicide" experiments can then be carried out to compare the effectiveness of disintegrations within these macromolecules in producing death. Such studies have been conducted previously in bacteria by Person (1) and by Rachmeler and Pardee (2).

In cultured mammalian cells, Puck and Yamada (3) and Marin and Bender (4) have carried out suicide experiments in cells growing at 37°C. Dewey, Humphrey, and Jones (5) have also used the "suicide" technique to study the efficiency of decay of tritium in various molecules at 37°C in inducing chromosome aberrations. Under the experimental conditions used in both of these studies, however, the normal metabolism of growing cells complicated the experiments since the labeled molecules did not necessarily remain in one place within the metabolizing cells; also, since cell division further complicated the picture, quantitative studies were difficult in these experiments. In order to overcome this difficulty, we incubated mammalian cells with low concentrations of tritiated compounds such that decay of tritium atoms did not have a significant effect on the cells. The cells which had incorporated ^3H were frozen and stored at −196°C. At this temperature, metabolic activity was virtually stopped but incorporated tritium atoms continued to decay. After permitting a sufficient number of tritium atoms to disintegrate, the cells were thawed and the per cent of cells surviving was estimated. Those cells which did not survive were killed primarily as a consequence of ^3H disintegrations. To ascertain the localization of ^3H-labeled molecules in the cells, radioautographs were made and chemical fractionations were carried out.

MATERIALS AND METHODS

Cell Line

The mouse leukemic cell line, L5178Y, was used in this study. The culture technique has been described in detail elsewhere as well as the characteristics of this cell line (6, 7).

Estimate of Survival

The per cent survival of the cells was estimated by the growth curve extrapolation method of Alexander and Mikulski (8). This method measures the growth potential of the cell population rather than the growth potential of individual cells as indicated by colony formation; however, it has been shown that both methods give somewhat parallel survival curves (9, 10). The per cent survival of labeled cells after a certain storage time was always estimated as the per cent of labeled cells which survived thawing immediately after freezing. The survival of these control cells was the same as that of the nonlabeled frozen cells.

Freezing, Storing, and Thawing

Cells growing exponentially in 1 and 2 liter flasks were concentrated to approximately 5×10^6 cells per ml and frozen slowly in the presence of Fischer's medium with 10% horse serum and 15% dimethylsulfoxide. After storage in liquid nitrogen for various periods of time, the cells were rapidly thawed in a 37°C water bath, and then added to 100 volumes of warm Fischer's medium. Details of the freezing procedure are reported elsewhere (11). The cell suspension was then distributed into 20-30 tissue culture tubes to obtain growth curves from which the per cent survival could be estimated.

It should be pointed out that the per cent survival of nonlabeled frozen cells was 76 ± 16% of that of nonlabeled nonfrozen cells (relative plating efficiency).

Labeling Cells with Tritiated Precursors

The method of labeling cells with thymidine-^{3}H has been described.[1]

In our experiments, tritiated 5'-uridine of high specific activity (20 Ci/mM, Schwarz Bio Research Inc., Orangeburg, N. Y., or New England Nuclear Corp., Boston, Mass.) was used. A concentrated suspension of cells was labeled with tritiated uridine in one of the two following ways: (*a*) the cells were labeled with 5'-^{3}H-uridine (10 μCi/ml) for 10 min; following incubation, the cells were washed three times with fresh warm Fischer medium containing 10^{-3} M uridine and then frozen by the method described above; (*b*) after pulse labeling with 5'-^{3}H-uridine (20 μCi/ml) for 10 min, the cells were washed and resuspended in fresh warm Fischer media for one population-doubling time (10 hr) before freezing.

Concentrated suspensions of cells in logarithmic growth phase were also labeled for 30 min with ^{3}H-*l*-lysine (50 μCi/ml and 100 Ci/mM) or with ^{3}H-*l*-histidine (100 μCi/ml and 4.7 Ci/mM, Schwarz Bio Research Inc.). The cells were then washed three times with fresh media and frozen in the manner described above.

Radiochemical Procedures

The intracellular distribution of tritium resulting from each type of tritium labeling procedure was determined by the method of Schmidt and Thannhauser (12) and of Schneider (13). Since the published procedures could not be applied to the present experiments, several modifications were introduced. The method finally selected for our experiments was as follows:

Approximately 1 ml of cold 10% TCA was added to test tubes containing previously washed and quick frozen cell pellets containing approximately 1–2 $\times$ 10^7 cells. The cells were kept at 4°C for 30 min and sedimented at the same temperature. The supernatant was saved as the acid soluble fraction. The sediment was washed twice with 3 ml of cold 1% TCA and centrifuged again. The supernatant, pooled with the previous supernatant, was considered to be the acid soluble fraction.

0.3 ml of 1 N NaOH was added to the sediment and incubated at 37°C for 30 min. The precipitate dissolved completely during this treatment. After incubation, the tubes were cooled to 0°C and one drop of 0.2% methyl violet indicator solution was added. Then, concentrated HCl was added dropwise to the solution to make the pH approximately zero. (The endpoint was the yellow-green color seen in 1 ml of 1 N HCl under the same conditions.) The solution was allowed to stand at 4°C overnight to allow a precipitate containing protein and DNA to form. The next day, the tubes were centrifuged and the supernatant containing hydrolyzed RNA was removed. The precipitate was washed three times with 1% TCA. The pooled, washed supernates contained the RNA fraction.

1 ml of 5% TCA was added to the remaining precipitate and placed in tubes in a boiling water bath for 15 min. The tubes were then centrifuged and the supernatant containing the DNA fraction was saved. The precipitate was washed once with 1 ml of 1% TCA and centrifuged. This supernatant was added to the DNA fraction and washed with 1% TCA. To achieve a clear separation of deoxyribonucleosides from protein, the precipitate was dissolved once more in 1 N NaOH at 37°C for 0.5 hr and acidified to pH 0 as before in the RNA fraction procedure. The tubes were stored at 4°C overnight to permit a precipitate to form. After centrifugation, the precipitate contained mostly protein and the supernatant was added to the DNA fraction.

The precipitate was dissolved by adding 1 ml of 1 N NaOH to tubes which were placed in

[1] Burki, H. J., and S. Okada. Killing of cultured mammalian cells by radioactive decay of incorporated tritiated-methyl-thymidine at −196°C. Submitted for publication.

a boiling water bath for 15 min. The solution formed thereby was designated the "protein fraction."

After chemical separation, the total tritium activity in each fraction was determined by: (*a*) adding a small aliquot (0.2 ml) to 15 ml of Bray's mixture (14) plus 1 ml of water in a glass scintillation vial, (*b*) counting the vials in a scintillation counter (Nuclear-Chicago Model 722, Nuclear-Chicago Corp., Des Plaines, Ill.), (*c*) determining the absolute counting efficiency, using the channel ratio counting method (15) and quenched tritium standards (Nuclear-Chicago Corp.), and (*d*) calculating the disintegrations per min per 0.2 ml and then converting to the radioactivity per total volume of the particular fraction.

Enzymatic Separation

A second method of determining the distribution of tritium in macromolecular cell fractions consisted of estimating the tritium activity in the soluble and insoluble fractions after treating the cells with the enzymes, ribonuclease (RNase) and deoxyribonuclease (DNase). This method was similar to the method of Person (1) used for bacteria.

The procedures were as follows: cells were washed with 5% TCA and centrifuged. The supernatant (pH 6.4–8.4) was designated as the acid soluble fraction. The precipitate was neutralized by adding 0.1 N sodium hydroxide using a phenol red indicator. Then, one of the following procedures was used: (*a*) 0.1 ml of stock $MgCl_2$ (0.1 M $MgCl_2$ solution) and 1.0 ml of 100 μg/ml DNase (Calbiochem) in tris buffer (0.05 M) at pH 7.8 were added to the neutralized precipitate and incubated at 37°C for 2 hr. The solution was acidified to approximately pH 0 (as in procedure above) at 4°C and kept overnight at this temperature to allow a precipitate to form. The suspension was centrifuged and the supernatant was considered to be the "DNA fraction." The precipitate was washed three times with cold 1% TCA and the supernatant was pooled with the DNA fraction; (*b*) RNase (Calbiochem, Los Angeles, Calif.) at 80°C for 20 min was dissolved in tris buffer (0.05 M) at a concentration of 200 μg/ml, and heated to inactivate nay possible DNase. 1.0 ml of RNase and 0.1 ml of stock $MgCl_2$ (0.1 M $MgCl_2$) was added to the precipitate. The mixtures were incubated at 37°C for 2 hr and then acidified, centrifuged, and washed as in the DNase treatment. The pooled supernatant fraction was designated as the "RNA fraction." The precipitates in (*a*) and (*b*) were dissolved by boiling in 1 ml of 1 N NaOH for 1 hr. This fraction was termed the "residual fraction."

Determination of the Tritium Activity per Cell

In experiments with cells labeled with tritiated uridine, lysine, or histidine, the disintegrations per cell were determined in the following fashion: (*a*) the number of cells per ml was determined using a Coulter Counter (Coulter Electronics, Chicago, Ill.); (*b*) the radioactivity per ml of cell suspension was determined by boiling an aliquot of cells in sodium hydroxide for 1 hr and then determining the tritium activity per ml of cells; and (*c*) the tritium activity per cell was obtained by dividing the total activity determined in the boiled aliquot of cell by the total number of cells in the aliquot.

Radioautographic Procedure

Cells were fixed with a 3:1 absolute ethyl alcohol:glacial acetic mixture and air-dried on slides. The slides were then dipped in Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.), stored in light-tight boxes, developed, and stained with Giemsa stain (16).

Calculation of Killing Efficiency

In plotting survival curves, the D_{37} dose of ^3H disintegrations was considered as that dose which resulted in 37% survival of cells. The killing efficiency ((α) was calculated from

$$\alpha = \frac{1}{D_{37}}.$$

In other words, α is the probability of cell killing per one decayed tritium atom per cell.

RESULTS

Suicide Experiments in Cells Labeled with ^3H-uridine, ^3H-l-histidine and ^3H-l-lysine

When cells were pulse labeled with 5-^{3}H-uridine and frozen immediately, the survival curves were exponential with a killing efficiency of 0.0015 and a D_{37} of 650 disintegrations per cell (Fig. 1). When the cells pulse labeled with 5′-^{3}H-uridine were frozen after incubation for another 10 hr (one generation time) in the absence of uridine-^{3}H, the killing efficiency decreased significantly to 0.001 and the D_{37} fell to 1000 disintegrations per cell (Fig. 2). In cells labeled with ^{3}H-1-histidine and ^{3}H-*l*-

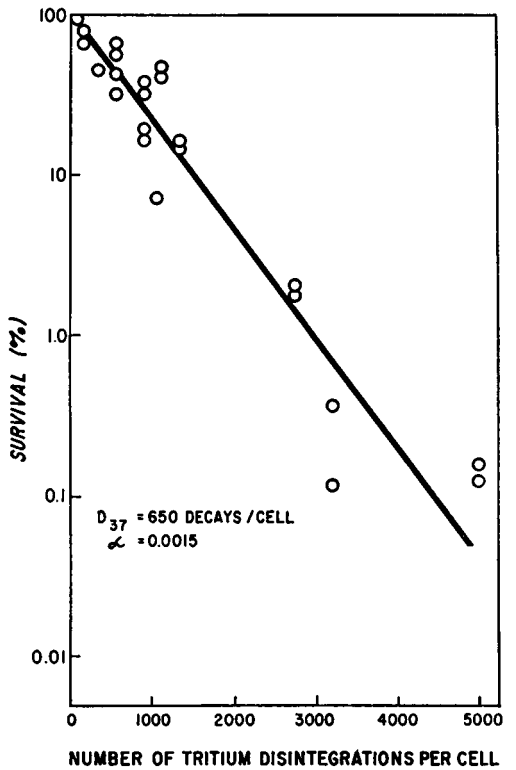

FIGURE 1 The survival curve of the cells labeled with 5′-^{3}H-uridine for 10 min. The data are taken from four experiments in which the rates of tritium disintegration were 114, 205, 356, and 652 disintegrations per cell per day during storage in liquid nitrogen. It should be added that the survival was independent of the disintegration rates.

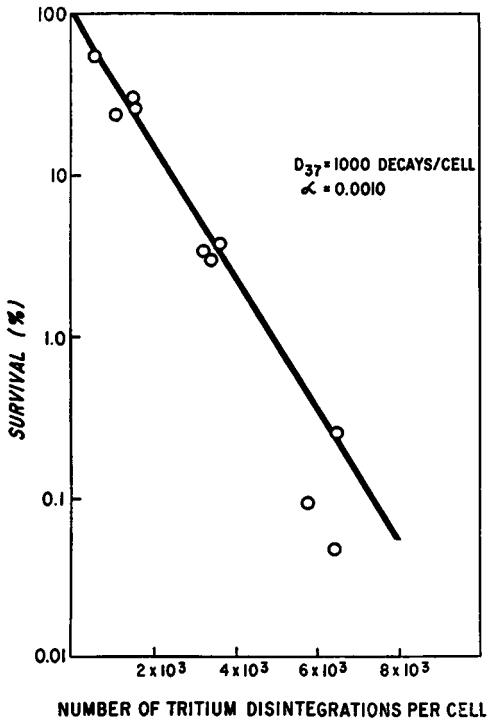

FIGURE 2 The survival curve of the cells pulse labeled with 5'-^{3}H-uridine and then chased with cold uridine for one population-doubling time. The fig. includes data from three experiments in which the rates of tritium disintegration were 59, 148, and 524 disintegrations per cell per day.

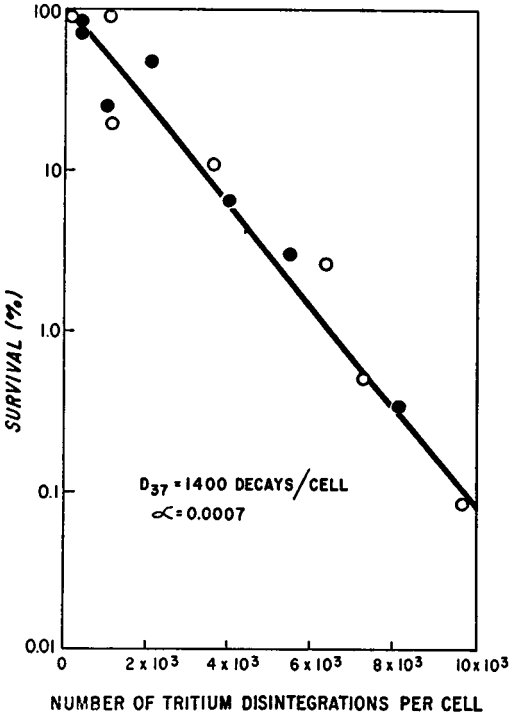

FIGURE 3 The survival curves of the cells labeled with ^{3}H-lysine and with ^{3}H-histidine. The open circles represent experiments using ^{3}H-lysine and the solid circles those using ^{3}H-histidine. The data were obtained in three experiments with ^{3}H-lysine in which the rates of tritium disintegration were 92, 195, and 490 disintegrations per cell per day. Three experiments were also carried out with ^{3}H-histidine in which the rates of tritium disintegration were 57, 280, and 504 disintegrations per cell per day.

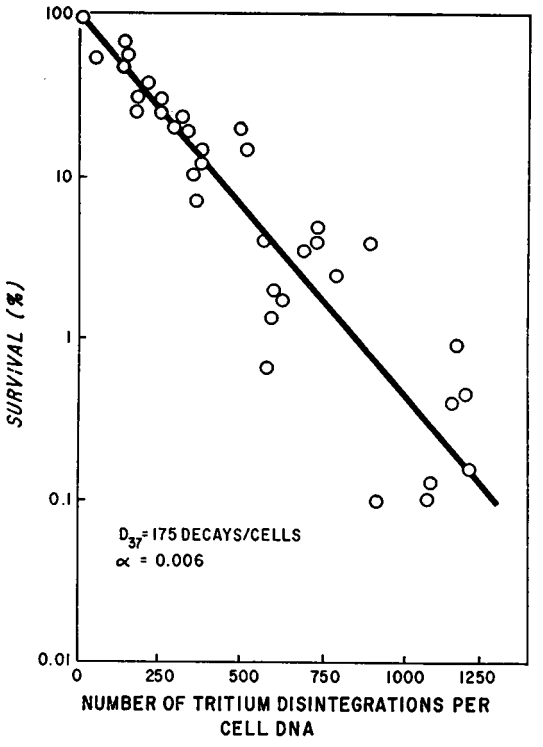

FIGURE 4 The survival curve of the cells labeled with ^{3}H-thymidine. (See footnote 1.)

lysine, the survival curves could be superimposed on each other and the D_{37} dose was further reduced to 1400 ^{3}H disintegrations per cell (Fig. 3).

In order to make further comparisons of killing efficiencies, Fig. 4 was prepared to show the survival curve of cells labeled with ^{3}H-thymidine for one generation.[1] The D_{37} dose was 180 disintegrations with a killing efficiency of 0.006.

Determination of Types of Tritium-Labeled Intracellular Molecules

The distribution of ^{3}H in four fractions (DNA, RNA, proteins, and acid soluble fraction) of the cells was determined for each type of labeling. Table I showed that most of the ^{3}H activity in ^{3}H-thymidine labeled cells was found in the DNA fraction. The ^{3}H activity of the cells pulse labeled with uridine was found to be divided mainly between the acid soluble and RNA fractions. The ^{3}H activity of uridine pulse labeled cells following incubation in nonlabeled media for one generation time was present for the most part in the RNA fraction, but also in the DNA fraction. In the experiments using ^{3}H-labeled amino acids, most of the ^{3}H activity was found in the acid soluble and some in the protein fraction.

Since the fractionation method of Schmidt, Thannhauser, and Schneider is by no means absolute, there is always a possibility of one fraction contaminating another with ^{3}H activity. In order to confirm the chemical fractionation studies, labeled cells

TABLE I

DISTRIBUTION OF TRITIUM ACTIVITY IN THE FRACTIONS OBTAINED BY THE MODIFIED METHOD OF SCHMIDT, THANNHAUSER, AND SCHNEIDER

Fraction	Per cent of total radioactivity in the cell				
	Thymidine-^{3}H Bifilar label*	Uridine-^{3}H Pulse labeled	Uridine-^{3}H Pulse labeled plus one generation time	Lysine-^{3}H	Histidine-^{3}H
Acid soluble	7.2	50.2	6.7	60.2	80.5
RNA	4.2	49.3	75.5	17.2	2.5
DNA	88.0	0.8	14.2	9.4	9.4
Residual (protein)	0.6	0.6	3.6	13.1	7.7

* Preliminary experiments in which the separation of DNA and protein fractions was not complete, indicated that the ^{3}H-thymidine distribution in unifilar and bifilar labeled cells in the acid soluble and RNA fractions was similar.

TABLE II

DISTRIBUTION OF TRITIUM ACTIVITY IN ENZYME DIGESTS

Fraction	Per cent of total activity of the cell		
	Thymidine-^{3}H	Uridine-^{3}H	Lysine-^{3}H or Histidine-^{3}H
Acid soluble	0.1	63.2	91.0
DNase soluble	99.3	—	2.0
RNase soluble	—	34.6	—
Residual (dissolved in NaOH)	0.6	2.2	7.0

were treated with deoxyribonuclease or with ribonuclease. The results are summarized in Table II. When the cells were labeled with tritiated thymidine, virtually all tritium radioactivity was located in the fraction digested by deoxyribonuclease. When cells were pulse labeled with tritiated uridine, most of the radioactivity was found in the acid soluble and ribonuclease-digested fractions. The tritiated amino acid labeling procedure resulted in radioactivity largely in the acid soluble fraction. These results are in good agreement with the chemical analysis.

Cellular Localization of Tritium Atoms

The intracellular distribution of tritium for each type of incorporated ^{3}H precursor was also determined by radioautography. In Fig. 5, one sees the subcellular distribution of tritium in cells pulse labeled for 10 min in tritiated uridine (*A*), and in cells pulse labeled for 10 min in tritiated uridine, and then incubated for 10 hr without uridine (*B*). From these radioautographs, it is clear that, after pulse labeling with

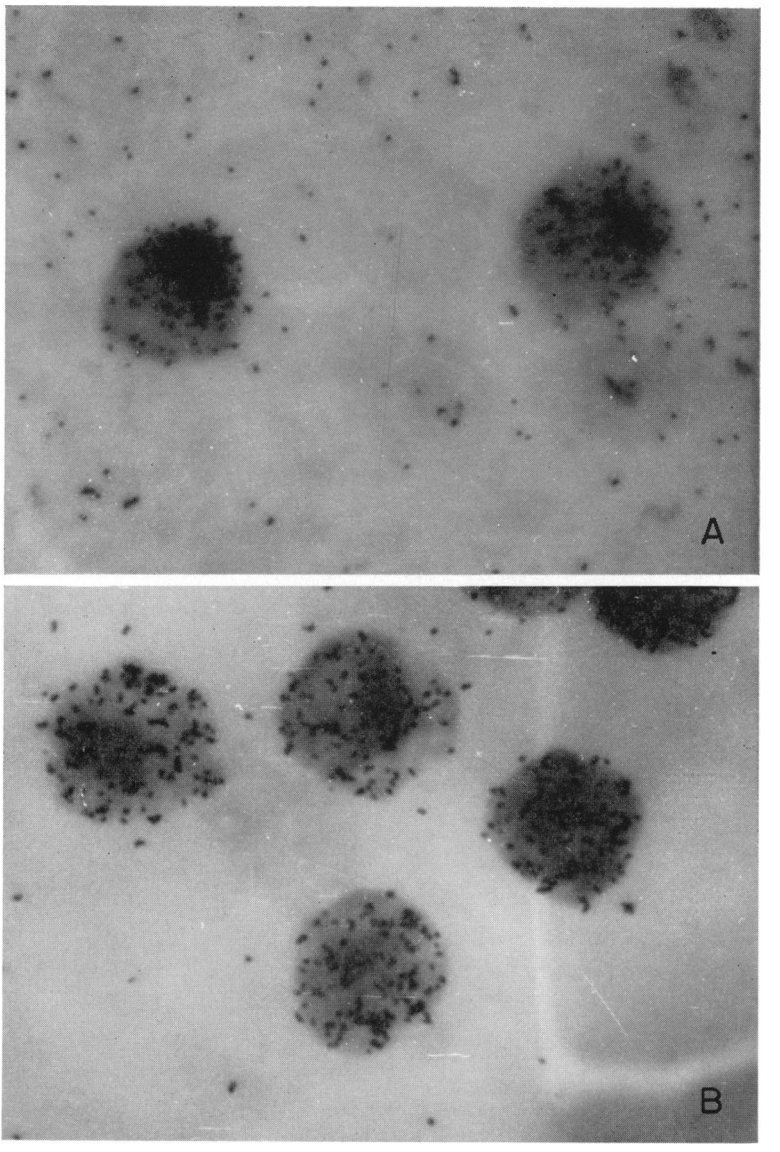

FIGURE 5 Radioautograph of the cells labeled with uridine-^{3}H. (*A*) The cells were pulse labeled for 10 min. (*B*) The cells were pulse labeled and chased with cold uridine for one population-doubling time.

uridine-^{3}H, most of the radio-activity was found in the nucleus. In the case of (*B*), tritium was distributed throughout the entire cell.

Fig. 6 shows the cellular localization of tritium atoms in the cells labeled with tritiated amino acids. The silver grains were distributed throughout the entire cell.

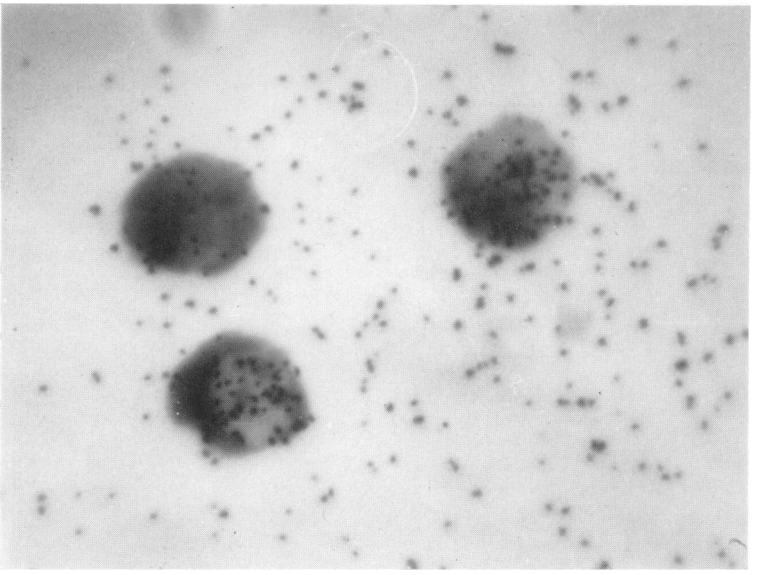

FIGURE 6 Radioautograph of the cells labeled with ^{3}H-lysine for 30 min.

Many grains were also seen between cells probably owing to the loss of the acid soluble fraction during the fixation of the cells.

DISCUSSION

The results of the present and previous experiments[1] are summarized in Table III. It is interesting to note that the exponential survival curves, except that of bifilar DNA labeling, allowed the D_{37} dose to be estimated in the units of ^{3}H disintegrations per cell. The D_{37} dose estimates suggest that ^{3}H decay in the nucleus is more efficient in killing the cells than that in the cytoplasm. In the nuclear labeled cells, the killing efficiency (0.0015) of the uridine-^{3}H labeled cells is one-quarter of that of thymidine-^{3}H unifilar labeled cells (0.006). If the killing is due mainly to decay of ^{3}H in the nucleus and if 50% of the incorporated tritium is in the nuclear RNA, the killing efficiency of uridine-^{3}H decay in the nucleus becomes 0.003, which is one-half of the killing efficiency of thymidine-^{3}H suicide. This suggests that ^{3}H decay in nuclear RNA (possibly messenger RNA, mRNA) is less efficient in cell killing than ^{3}H decay in DNA. In the two types of uridine-^{3}H labeled experiments, the observed differences in killing efficiency might be attributed to less efficient killing of cells by ^{3}H decay in transfer RNA (tRNA) and ribosomal RNA (rRNA) than that in mRNA. Thus, the effectiveness of tritium disintegrations occurring at different molecular sites can be noted as follows: DNA > mRNA > tRNA, rRNA > amino acids. The same relative order of efficiency of killing after labeling with similar precursors has been reported previously by Puck and Yamada (3) and in Chinese

TABLE III

SUMMARY OF "SUICIDE" DATA IN L5178Y CELLS

Type of labeling	Location of tritium activity	D_{37}*	Killing‡ efficiency (α)	Ratio of α over α of the cells with unifilar labeled DNA
Thymidine-^{3}H label for one generation time	Unifilar DNA	175	0.006	1.0
Thymidine-^{3}H label for four generation times	Bifilar DNA	D_o = 80 D_Q = 250	0.0125	2.2
Thymidine-^{3}H label for four generation times plus one generation time with cold thymidine	(reverse) Unifilar DNA	165	0.006	0.94
Uridine-^{3}H pulse labeled	Nuclear RNA	650	0.0015	0.27
Uridine-^{3}H pulse labeled plus one generation time growth in cold media	Whole cell RNA	1000	0.0010	0.18
Histidine-^{3}H or Lysine-^{3}H	Whole cell acid soluble and protein fraction	1400	0.0007	0.13

* Number of disintegrations of tritium atoms/cell.

‡ $1/D_{37} = \alpha$.

hamster cells by Marin and Bender (4). In bacteria, Rachmeler and Pardee (2) and Person (1) found the killing efficiency relationships of different tritiated precursors to be similar to the results reported above. It should be added that although difference of killing efficiencies among various labeled molecules represents the sum contributions of various factors, such as beta-irradiation of the nucleus, damage to labeled molecules by transmutation and recoil nuclei, etc., it is not possible to assess quantitatively the role of each in killing in the present experiments because of heterogeneity of subcellular distribution of ^{3}H atoms and because of the difference in the types of ^{3}H-labeled molecules.

Finally, it should be added that this relationship does not necessarily hold for other biological effects. In Chinese hamster cells, thymidine-^{3}H was as effective in ^3H_2O as in the nucleus in producing chromosome aberrations (5). Similarly, in *E. coli*, uridine-^{3}H was found to be more effective in the induction of mutations than thymidine-^{3}H (17).

This paper was supported by a U.S. Atomic Energy contract (AT(30-1)-1286) and partially under contract with the U.S. Atomic Energy Commission at the University of Rochester Atomic Energy Project. It has been assigned Report No. UR-49-834.

Received for publication 22 September 1967.

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